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Clinical and Experimental

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VOL. 45 MAY, 1955 NO. 5

CLINICAL AND EXPERIMENTAL

THE RADIOACTIVE (I^{131}-TAGGED) ROSE BENGAL UPTAKE-EXCRETION TEST FOR LIVER FUNCTION USING EXTERNAL GAMMA-RAY SCINTILLATION COUNTING TECHNIQUES

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WITH THE TECHNICAL ASSISTANCE OF PATRICIA PEEL AND RHODA DEVICK

INTRODUCTION

THE radioactive rose bengal test described in this report has a number of advantages over many other methods of testing liver function. First, it measures polygonal cell function directly rather than by changes occurring in the blood (thereby eliminating repeated venipunctures); second, it registers graphically the rate of dye uptake and time necessary for its excretion from the liver; third, it is the only dye test which can be used safely in the presence of biliary tract obstruction because of the minute quantities and low toxicity of the dye employed; fourth, it gives information concerning liver circulation as well as patency of the biliary tract, and fifth, it appears to be many times more sensitive than nonradioactive dye tests.

Although (nonradioactive) rose bengal has been used for many years as a dye to test liver function, these additional advantages are attainable because the chemical structure of the rose bengal molecule (the potassium salt of tetra-iodo-tetra-chlor-fluorescene) permits replacement of the stable iodine atoms with radioactive iodine (I^{131}) atoms which emit gamma rays. Following intravenous injection, the radioactive dye uptake may be detected over the liver

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area externally by using suitable scintillation counting equipment. This technique gives a dynamic record of the capacity of the liver to remove the dye from the blood stream and excrete it into the bile passages.[1]

The purposes of this report are (1) to describe the techniques of the radioactive rose bengal test as developed in animal studies and the necessary modifications when it is applied clinically; (2) to demonstrate from the results of experiments in rabbits the high sensitivity and low toxicity of the test and its potential clinical applicability; (3) to present typical clinical results which confirm the animal studies and also demonstrate the usefulness of the test in evaluating liver functions.

MATERIAL AND EQUIPMENT

The rose bengal used (C.I. 779) is rendered radioactive by a procedure utilizing ion-exchange reactions giving a high specific activity, i.e., about 1 mc. per milligram of dye.[2] The I^{131} appears to be firmly integrated into the dye molecule, since only negligible amounts of radioactivity can be detected in thyroid tissue of rabbits 24 hours following intravenous injection of 30 μc of the radioactive dye. The rose bengal is supplied in a solution containing 10 mg. of dye and 10 mc. of I^{131} per milliliter. A working solution containing 15 μc per milliliter is prepared by making suitable dilutions of the tagged dye in 1 per cent non-radioactive rose bengal.

Virgin female Dutch rabbits, weighing 1,250 to 1,750 grams, were used exclusively. Gamma radioactivity was measured and recorded over a hind limb for estimating the rate of blood clearance, and over the liver area for recording the rates of uptake and excretion from this organ. These measurements were made with a collimated gamma-ray scintillation counter[3] connected through a rate meter to an Esterline Angus Recorder (Fig. 1).

PRELIMINARY STUDIES IN RABBITS

In initial investigations, where a 1 per cent (W/v) solution of nonradioactive rose bengal was used, a satisfactory blood clearance curve could be obtained with a dosage of 10 mg. per kilogram of body weight (average dose per animal was 15 mg. of dye).

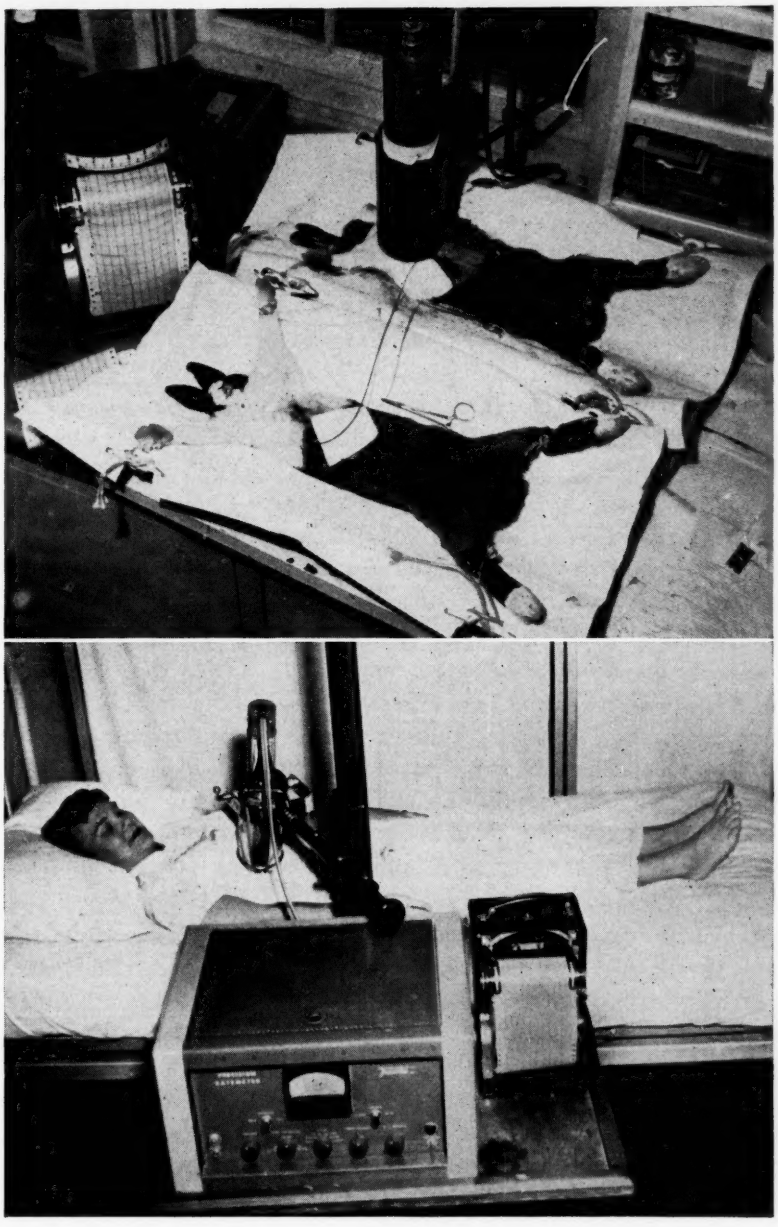
Samples of whole blood obtained at 2, 5, 8, and 15 minutes following intravenous injection of the dye solution were treated with 5 volumes of 95 per cent ethyl alcohol, and the precipitates were removed by centrifugation. The supernatant solutions were analyzed colorimetrically for dye content using a Beckman Model DU Spectrophotometer. The dye concentration found in the two-minute (mixing time) sample was considered as 100 per cent and the coefficient of clearance was computed from the dye content of the subsequent samples. Parallel blood clearance studies, performed in normal and chloroform-poisoned animals to compare Bromsulfalein and rose bengal, yielded similar curves for both dyes, confirming the recent work of Cohen and associates.[4, 5]

TECHNIQUE FOR THE RADIOACTIVE ROSE BENGAL TEST IN RABBITS

An animal is anesthetized with Nembutal and restrained in the supine position. The lead collimated gamma-ray scintillation counter (calcium tungstate crystal)[3] is placed over the liver area and positioned in relation to the xiphoid process and the margin of the rib cage on the right side. The sensitive element of the counter is kept withdrawn into the

lead collimator and the counter head is tilted at a 20 degree angle toward the chest. (This procedure helps to ensure measurement of gamma-ray activity in the liver primarily and minimizes interference from radioactive dye excreted into the gall bladder and gastrointestinal tract.)

a.



b.

Fig. 1.—*a*, Arrangement of apparatus for performing radioactive (I^{131}) rose bengal liver uptake-excretion test in rabbits (bile transfer-reabsorption experiment).

b, Arrangement of apparatus for performing radioactive (I^{131}) rose bengal liver uptake-excretion test in human subjects.

The test dosage of labeled rose bengal used throughout contained 20 mg. of dye and 30 μc of I^{131} per animal, an amount sufficient to produce an increase of 500 to 750 counts per second above background levels. Injections were made into a marginal ear vein at a rate of approximately 0.1 ml. per second.

EXPERIMENTAL RESULTS

I. *Typical Findings in Rabbits With and Without Liver Disease.*—Tracings shown in Fig. 2 demonstrate typical patterns of liver uptake and excretion obtained by injecting 30 μc of radioactive rose bengal intravenously in normal rabbits, compared with animals previously given carbon tetrachloride poisoning or having complete biliary tract obstruction from surgical ligation of the common bile duct.

The liver uptake curve in normal rabbits reaches a maximum in 10 to 25 minutes, and is reciprocally related to the rate of blood clearance with respect to timing. The peak level of activity over the liver represents a transitory equilibrium between rates of liver uptake and excretion. The slope of the curve following the plateau indicates primarily the rate at which the liver is capable of excreting the dye via the biliary tract, since the blood is essentially cleared at this time, and there is virtually no additional liver uptake. This excretion curve is linear in most instances for 10 to 20 minutes and then changes its slope gradually. The break away from linearity appears sooner if the counter is not directed away from the gall bladder and the upper gastrointestinal tract areas. It is likely that the liver excretion curve would have a more constant slope if it were possible to obtain better collimation over the liver area. Such problems of geometry are much less difficult in man because of liver size.

II. *Studies on Absorption of Radioactive Rose Bengal From the Gastrointestinal Tract.*—First, several rabbits were intubated surgically and measured amounts of I^{131}-tagged rose bengal were instilled directly into the duodenum. Liver uptake of the dye was measured by external gamma-ray scintillation techniques for four to six hours. In none of these animals could any significant activity be detected over the liver area. In addition, on sacrifice, liver tissue was found to be almost completely free of radioactivity (background levels).

Second, to determine whether I^{131}-tagged rose bengal that has passed through the liver is absorbable from the gastrointestinal tract, one rabbit was injected intravenously with radioactive dye and the bile was shunted from the duodenum of this animal to the duodenum of a second rabbit (Fig. 1, *a*). The absorption of rose bengal-bile mixture was tested by recording the gamma-ray activity of the second animal for several hours. No significant increase of radioactivity could be detected over the liver area, although the radioactivity over the gastrointestinal tract rose to a level indicating that by the end of three hours, approximately one-third of the injected 30 μc dose was present in the duodenum of the second animal. Furthermore, assay of liver tissue from the recipient animal, sacrificed at the end of five hours, showed insignificant amounts of gamma-ray activity. Thus, it appears that rose bengal, both in aqueous solution and mixed with bile after passage through the liver of one rabbit, is not absorbed from the gastrointestinal tract of normal rabbits.

III. *Studies on Toxicity of Rose Bengal in Rabbits.*—Nonradioactive rose bengal was administered intravenously to rabbits in massive doses, viz., 20 ml. of a 1.0 per cent aqueous solution of the dye (or about 130 mg. per kilogram).

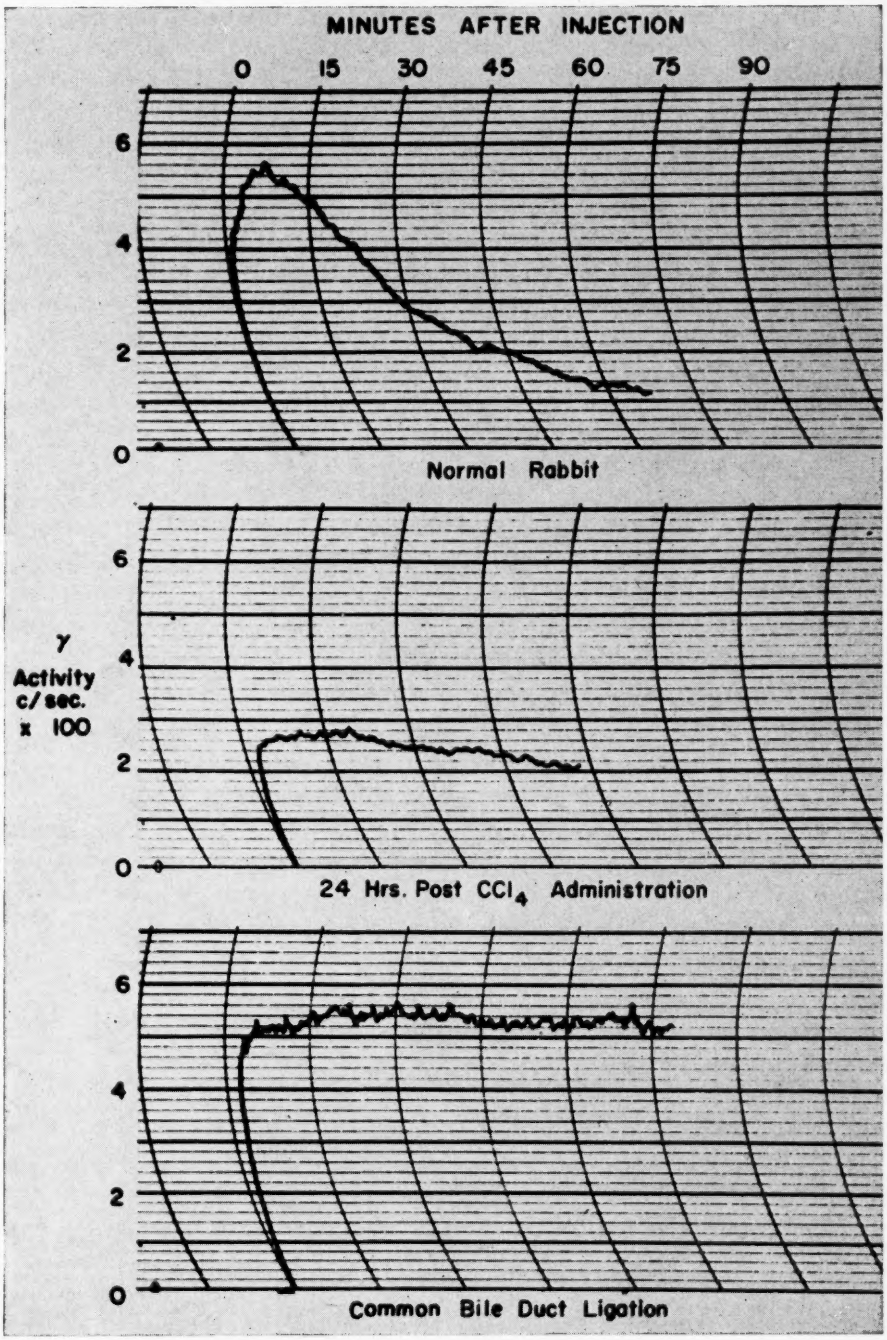


Fig. 2.—Typical rose bengal liver uptake-excretion patterns in normal and abnormal rabbits.

No detectable acute or delayed toxic effects were noted during a 14-day observation period. However, large doses of dye (in the 50 to 100 mg. range) require several hours for a 20 to 30 per cent excretion from the liver.

In anticipation of clinical circumstances where the usual 100 mg. dose of dye might be toxic in patients because of either extensive liver damage or biliary tract obstruction, the minimum amount of I^{131}-tagged dye needed to give a satisfactory tracing in normal animals was determined. A dosage of 30 μc of radioactivity contained in 0.25 to 1.0 mg. of dye gave tracings indistinguishable from those obtained with the standard 20 mg. dose.

IV. *Sensitivity of the Radioactive Rose Bengal Test for Detecting Liver Poisoning From Carbon Tetrachloride and Chloroform.*—Several groups of rabbits were poisoned with single doses of decreasing amounts of carbon tetrachloride and chloroform administered by gastric intubation, ranging from more than 1 Gm. to 16 μg per kilogram. Rose bengal liver uptake and excretion patterns were determined at specific intervals following the administration of the chorinated hydrocarbon. Sections were prepared from microscopic examination, and aliquot specimens of liver tissue were assayed for total fat content by the method of Delsal.[6]

TABLE I. EARLIEST EFFECTS (0 TO 7 HOURS) OF ACUTE CARBON TETRACHLORIDE POISONING IN RABBITS ON THE LIVER UPTAKE AND EXCRETION OF RADIOACTIVE ROSE BENGAL

TIME AFTER POISONING* (HOURS)	NUMBER OF ANIMALS	MAXIMUM AMPLITUDE OF LIVER UPTAKE (COUNTS/SEC.–BACKGROUND)	RATE OF LIVER CLEARANCE† (COUNTS/SEC./MINUTE)
0 (Normal)	12	480	12.3
1	1	540	3.2
3	1	550	3.5
4	1	440	0.0
5	1	410	4.1
7	1	370	4.6

*An 840 mg. per kilogram dose of carbon tetrachloride was administered in olive oil via gastric intubation to all rabbits. Normal (control) animals given plain olive oil in same manner.

†Rate of liver clearance—expressed as the decline of γ-radioactivity (c/s—bkg) per minute of observation time over the liver.

TABLE II. LATER EFFECTS (1 TO 7 DAYS) OF ACUTE CARBON TETRACHLORIDE POISONING IN RABBITS ON THE LIVER UPTAKE AND EXCRETION OF RADIOACTIVE ROSE BENGAL

TIME AFTER POISONING* (DAYS)	NUMBER OF ANIMALS	MAXIMUM AMPLITUDE OF LIVER UPTAKE (COUNTS/SEC.—BACKGROUND)	RATE OF LIVER CLEARANCE† (COUNTS/SEC./MINUTE)
0 (Normal)	12	480	12.3
1	4	300	0.84
2	4	310	3.83
4	4	260	2.68
7	4	270	3.52

*An 420 mg. per kilogram dose of carbon tetrachloride was administered in olive oil via gastric intubation to all rabbits. Normal (control) animals given plain olive oil in same manner.

†Rate of liver clearance—expressed as the decline of γ-radioactivity (c/s—bkg) per minute of observation time over the liver.

Results of such studies in more than 45 rabbits have provided the evidence for the following general statements: (1) definite abnormalities of liver uptake-excretion patterns for tagged rose bengal can be detected as soon as one hour after an 840 mg. per kilogram dose of carbon tetrachloride (Table I); (2) the pattern becomes grossly abnormal by 24 hours, and does not return to normal for at least 7 days (Table II); (3) the first detectable abnormality of the curves is a decreased excretion rate followed by a lowered amplitude of the maximum uptake value; (4) recovery is manifested by improvement of the excretion slope and by increase of the amplitude of the uptake curve; (5) histologically, the degree of fatty degeneration of the polygonal cells roughly parallels the change in maximum amplitude of the dye uptake curve. However, the excretion segment of the curve becomes depressed even before fatty change is evident microscopically; (6) groups of animals given doses of carbon tetrachloride as small as 16.0 μg have shown definitely abnormal patterns within 24 hours (Table III).

TABLE III. SENSITIVITY OF BROMSULFALEIN BLOOD RETENTION TEST COMPARED TO RADIOACTIVE ROSE BENGAL LIVER UPTAKE-EXCRETION TEST FOR ESTIMATING LIVER DAMAGE IN RABBITS POISONED WITH CARBON TETRACHLORIDE

	BROMSULFALEIN TEST		RADIOACTIVE ROSE BENGAL TEST‡		
DOSAGE OF CCl_4* (MG./KG.)	NUMBER OF ANIMALS	BLOOD RETENTION AT 30 MINUTES† (MG. PER CENT)	NUMBER OF ANIMALS	AMPLITUDE OF LIVER UPTAKE	RATE OF LIVER CLEARANCE
1116	1	27.0			
1041	0		1	Markedly depressed	Markedly decreased
857	0		1	Depressed	Markedly decreased
452	0		1	Depressed	No clearance
428	0		2	Depressed	Markedly depressed or absent
324	2	11.15	0		
221	0		1	Depressed	No clearance
143	1	17.0	0		
126	0		1	Near normal	Decreased
79	1	3.0	0		
63.6	0		1	Near normal	Decreased
32.4	1	3.5	0		
26.6	0		1	Near normal	Depressed
12.7	0		1	Depressed	Near normal
7.9	0		3	Near normal	Moderately depressed
3.24	1	3.5	0		
1.59	0		1	Depressed	Near normal
0.324	4	2.0	0		
0.159	0		3	Near normal	Near normal
0.0159	0		1	Near normal	Depressed
0.0000	3	2.6	12	Normal	Normal

*The carbon tetrachloride was administered in olive oil via gastric intubation to all animals 24 hours prior to performing the tests.

†Normal value for Bromsulfalein blood retention at 30 minutes is 4 mg. per cent or less. Dosage of Bromsulfalein used was 5 mg. per kilogram.

‡The patterns of rose bengal liver uptake and excretion in poisoned animals were evaluated by comparison with tracings obtained on normal (control) rabbits (given plain olive oil in the same manner) tested simultaneously.

In animals given various doses of carbon tetrachloride and tested with Bromsulfalein blood clearance techniques 24 hours after poisoning, no increase

in dye retention occurred in the 30-minute sample from animals receiving doses of hydrocarbon less than 143 mg. per kilogram. These experiments demonstrate that the radioactive rose bengal test is highly sensitive (many times more sensitive than the Bromsulfalein blood clearance test) in indicating functional impairment of the liver from poisoning by carbon tetrachloride (Table III).

V. *Effect of Reticuloendothelial System Blockade on the Pattern of Rose Bengal Uptake and Excretion.*—Since early investigators of this dye[7-10] indicated that its excretion by the liver involved the reticuloendothelial system, our original interest in rose bengal was to study this dye as a possible substitute for colloidal prodigiosin in testing phagocytic function of the reticuloendothelial system.[1] Several groups of six rabbits each, treated with intravenous injections of reticuloendothelial system blocking agents such as colloidal thorium dioxide, India ink, hemolyzed erythrocytes, and gelatin, as well as a group which had received 1,000 r whole body x-irradiation 7 to 10 days previously, were all tested with I^{131}-tagged rose bengal (Table IV).

TABLE IV. FAILURE OF RETICULOENDOTHELIAL SYSTEM BLOCKING AGENTS TO ALTER THE LIVER UPTAKE-EXCRETION PATTERN OF RADIOACTIVE ROSE BENGAL

BLOCKING AGENT	DOSAGE	NUMBER OF ANIMALS	RADIOACTIVE ROSE BENGAL RESULTS	
			AMPLITUDE OF LIVER UPTAKE	RATE OF LIVER CLEARANCE
Colloidal thorium dioxide	2 ml./kg.	6	Normal	Normal
Distilled water to hemolyze erythrocytes	5 to 20 ml.	6	Normal	Normal
Gelatin (5% solution)	33 mg./kg.	1	Normal	Normal
India ink (Higgins)	1 ml./kg.	6	Normal	Normal
1:5 dilution	1,000 r (7 days previously)	3	Normal	Normal
X-irradiation (whole body)	1,000 r (10 days previously)	3	Normal	Normal

Note: All blocking agents were given intravenously 1 to 2 hours before the test, except for the x-radiation exposure.

Although phagocytic function of the reticuloendothelial system has been shown to be significantly depressed in such radiated or "blocked" rabbits,[1] none of the animals in any of these groups showed abnormal radioactive rose bengal liver uptake-excretion patterns. Thus, our results indicate that rose bengal uptake and excretion are both functions exclusively of the polygonal cells of the liver, and that the reticuloendothelial system has little, if any, part in the passage of this dye through the liver and biliary tract of rabbits. These findings in rabbits confirm the recent reports of Cohen and associates,[5] which indicate that the blood clearance curves for rose bengal and Bromsulfalein are nearly identical in patients having normal hepatic function.

CLINICAL STUDIES WITH THE RADIOACTIVE ROSE BENGAL LIVER FUNCTION TEST

I. *Modifications in Technique of the Test for Application in Patients.*—After trying various positions, degrees of collimation, dye concentrations, and amounts of radioactivity, the following procedure was established tenatively, and gave reliable as well as reproducible

results: the lead collimated counter (sodium iodide crystal) is positioned on the right anterolateral aspect of the chest wall, over the upper margin of the liver, as determined by percussion, and directed away from the gall bladder area (Fig. 1, *b*). The sensitive element

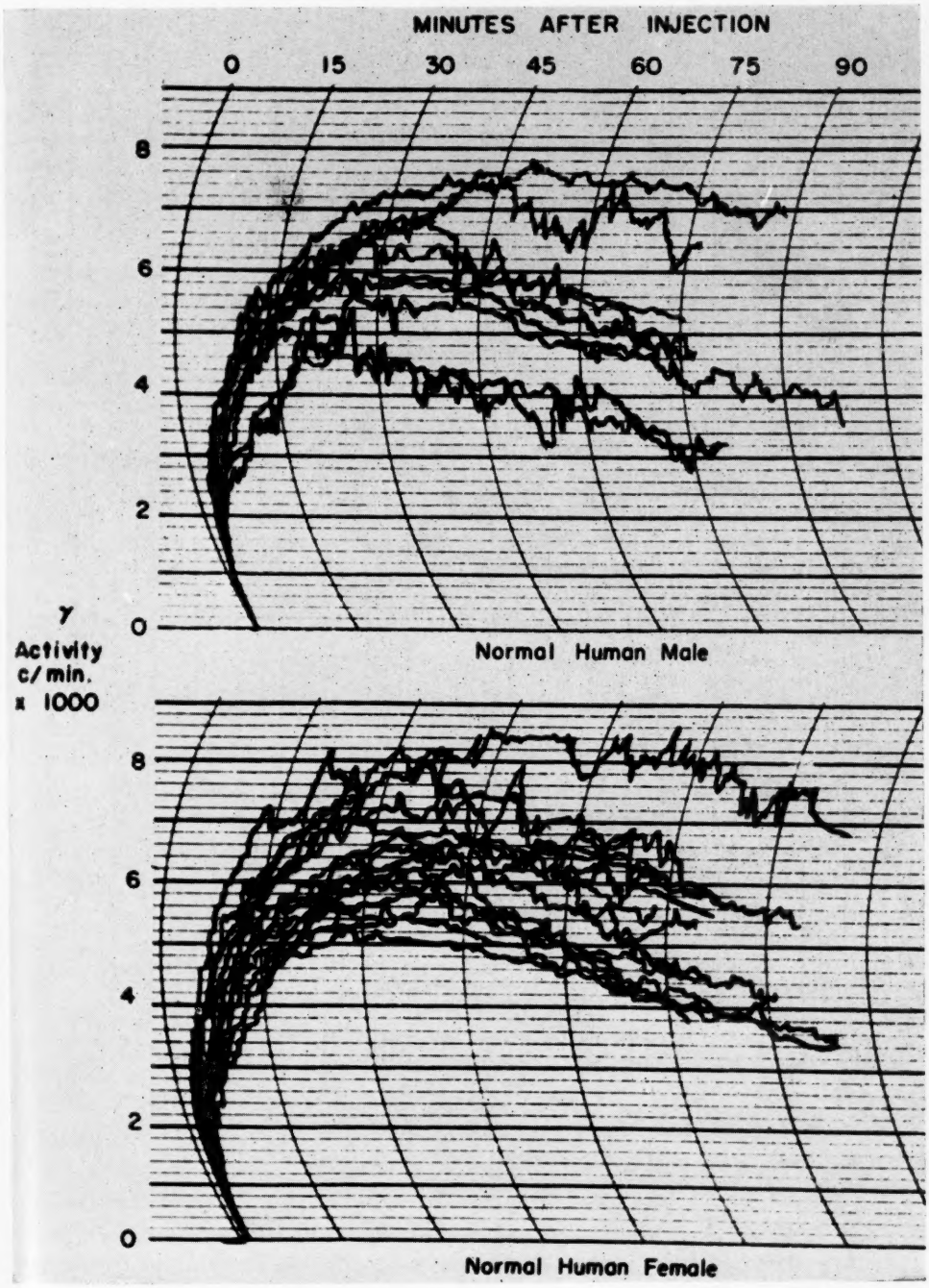


Fig. 3.—Composite (superimposed) tracings of radioactive (I^{131}) rose bengal liver uptake and excretion in normal human subjects.

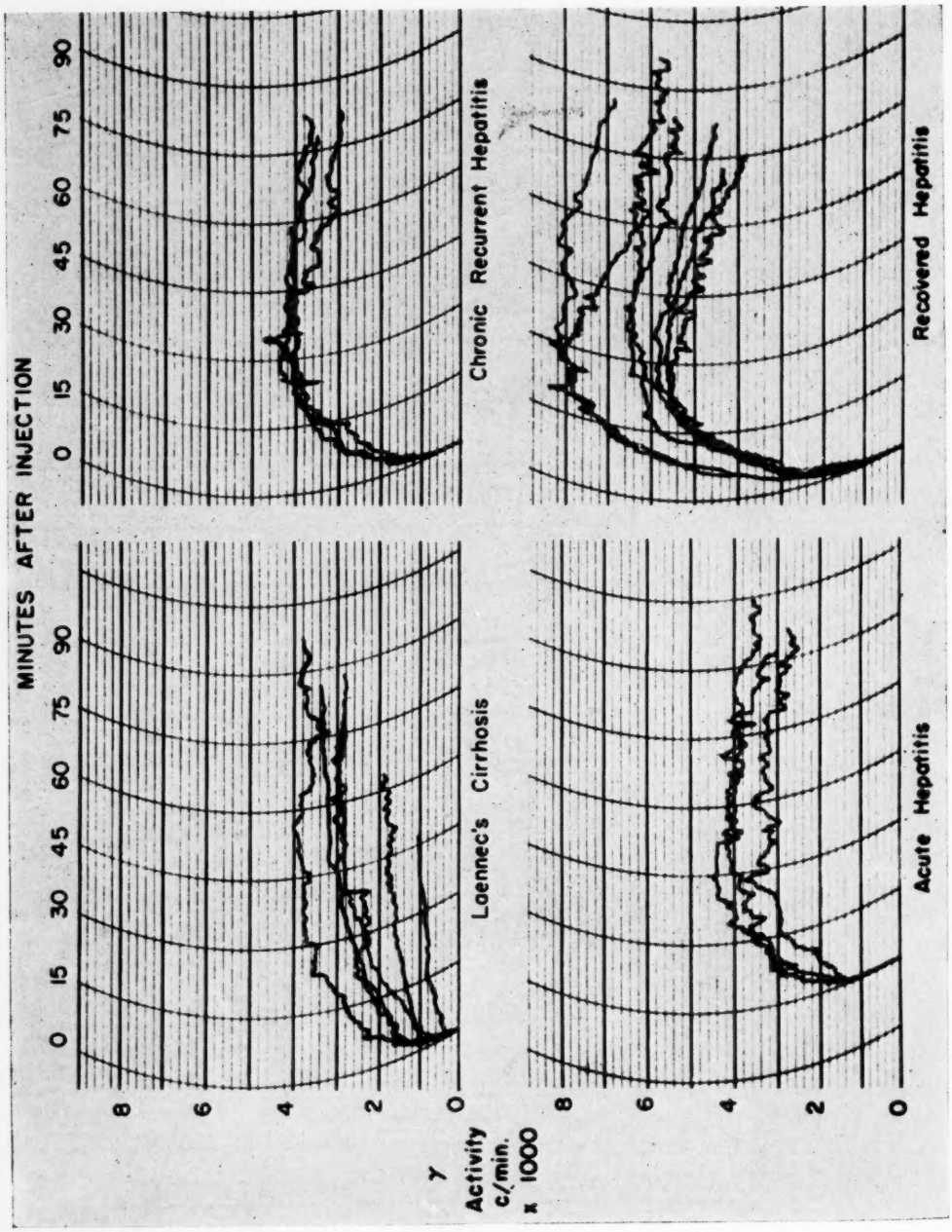


Fig. 4.—Composite (superimposed) tracings of radioactive (I^{131}) rose bengal liver uptake and excretion in patients with known liver disease.

is withdrawn a distance of 6 inches into the lead collimating shield. With the rate meter set to register a range of 10,000 counts per minute, a 1 μc antimony standard button held at the end of the counter gives 80 per cent of full-scale deflection on the Esterline-Angus Recorder. The 10-second time constant is used to obtain a tracing relatively free from random fluctuations.

The dosage of dye used in this preliminary series of sixty patients has varied between 0.5 and 5.0 mg., and the amount of radioactivity has been held constant at 5 μc by suitable dilutions of the I^{131}-tagged dye sample with either saline solution or a saline solution of the nonradioactive dye. The test dose (volume 2 to 5 ml.) is injected intravenously at a rate of approximately 0.1 ml. per second.

The patient is instructed to remain in a reclining position for the duration of the test; however, it is important only that no significant change occur in the position of the counter and the chest wall. Movement of upper and lower extremeties is permissible. The tests have been performed in the fasting and nonfasting states, and no significant differences have been noted in the same patients tested both ways. A satisfactory uptake and excretion pattern can be obtained in normal individuals in a period of 60 to 90 minutes. More time is required to plot the curve in patients having severe liver impairment. To date, there have been neither acute nor delayed type reactions to the labeled dye. Several patients have been tested repeatedly without detectable ill effects.

II. *Results in Normal Individuals and Patients With Proved Liver Diseases.*—Prior to performing the test in patients with hepatic disease, the pattern of liver uptake and excretion in normal individuals was established from the tracings of nine men and seventeen women having no history or physical evidence of impaired liver function. In general, the curves for men and women are nearly identical, except that in women the average amplitude is 10 to 20 per cent greater (probably related mainly to differences in liver weight and size). For superimposed copies of the actual tracings, see Fig. 3. Thus, after establishing the limits of the pattern in normal men and women, the test was performed on two subjects daily; the first, a person with normal liver function, and the second, a patient with known liver disease. The patients with liver disease were selected for study after thorough clinical and laboratory investigation, including liver biopsy in some cases. Patients with advanced portal cirrhosis and severe acute hepatitis were tested first; later, patients with subsiding hepatitis, chronic recurrent hepatitis, and some who had apparently recovered completely from a single episode of hepatitis were tested, using the same procedures.

Tracings shown in Fig. 4 demonstrate the typical patterns obtained in testing patients with advanced portal cirrhosis compared with others having less severe liver impairment. The tracings of subjects with both acute and chronic hepatitis deviate from normal in one or more respects. The uptake slopes are depressed and the amplitude of the peaks is generally lower than in normal individuals. In some instances, the excretion rates are impaired. Several patients with chronic and/or recurrent hepatitis show definitely abnormal curves. In the latter cases, various other liver function tests also indicate persistent hepatic damage and these individuals have symptoms and/or signs of liver disease. The patterns of liver uptake-excretion in seven patients, who clinically have recovered from acute hepatitis, are normal in nearly all respects (Fig. 4).

Fig. 4.—Composite (superimposed) tracings of radioactive (I^{131}) rose bengal liver uptake and excretion in patients with known liver disease.

DISCUSSION

We are cognizant of the complexity of the problems involved in evaluating liver functions generally.[11, 12] Most tests indicate disturbance in only one of the numerous functions of the liver. It is well known that much of the liver mass may be destroyed before functional impairment is detectable by routine tests. Some of the more sensitive tests have the disadvantage of giving positive reactions in the absence of liver disease. On the basis of our available laboratory and clinical data, it appears that the radioactive rose bengal liver uptake and excretion test may have wide applicability in general medicine; however, further investigations are needed to clarify and quantitate the interpretation of the tracings obtained.

It is possible that further modifications in technique may be advantageous. For example, to obtain tracings only on the rate and amount of liver uptake of the dye, a wide-angle scintillation counter (without much collimation) would be adequate, thus permitting the use of smaller doses of dye and less radioactivity. Such a test could probably be completed in 20 to 30 minutes on most subjects and would provide much of the essential information, except for data on the excretory ability of the liver cells and patency of the biliary passages. It would be useful mainly in testing patients with hepatitis, cirrhosis, toxemias of pregnancy, or liver poisoning, where obstruction of the biliary tract is not suspected from the clinical picture.

Another potential improvement might be to compute the dosage of both dye and radioactivity on the basis of body weight. (Present data indicate that the main reason for the higher amplitude of the tracings in women compared with men is due to the mean difference in liver weight and size.) Furthermore, it is entirely possible to decrease the amount of radioactivity to about 1.0 μc by slight reduction in collimation of the counter and by changing the activity range (counts per minute) of the rate meter.

Another aspect of the test needing further study is related to the interpretation of the initial uptake segment of the curve. By speeding the recorder during the first few minutes following dye injection, it may be possible to measure arm-to-liver circulation time and mixing time, as well as to determine accurately the base-line blood level of gamma-ray activity over the liver, from which reference point the true rate of dye uptake by the liver may be calculated. Available data indicate that the uptake patterns during the first few minutes may register significant differences between normal individuals and those having severe liver disease, as well as reflect alterations of circulatory dynamics within the liver. This phase of the problem is being studied by Dr. Leslie R. Bennett under a separate project, in cooperation with us.

One more avenue for investigation is suggested by our findings that large doses of dye require several hours for as little as 20 to 30 per cent excretion from the liver. These observations indicate that a priming dose of nonradioactive rose bengal might be used prior to, or administered simultaneously with, the tagged dye to obtain a relatively constant and prolonged (one- to two-hour)

level of gamma-ray activity in the liver. This procedure would give adequate time for careful scanning of the anterior liver surface with a collimated counter to locate large liver tumors, cysts, or abcesses.

The radioactive rose bengal liver uptake-excretion test may be performed with equipment and techniques similar to those employed for the radioiodine uptake test for thyroid function. The basic studies in rabbits and the preliminary results in patients with radioactive rose bengal have encouraged us to believe that this test may be developed into a standard clinical procedure. The single dynamic tracing obtained by recording γ-ray activity directly over the liver provides information from which hepatic circulation, polygonal cell function, and biliary tract patency may be evaluated.

SUMMARY

Laboratory studies in rabbits indicate the potential clinical applicability, high sensitivity, and relative safety of the radioactive rose bengal liver uptake-excretion test for determining the presence of liver and biliary tract diseases. The techniques employed in rabbits and the modification required for application in clinical medicine are described. Preliminary clinical results demonstrate that patients with some of the common diseases of the liver have fairly typical uptake-excretion patterns which are readily distinguishable from those recorded in normal individuals.

Need for additional clinical investigation is stressed, and some possible advantages from further modifications in technical procedures are suggested.

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A NEW ANTIRABIES VACCINE FOR HUMAN USE

Clinical and Laboratory Results Using Rabies Vaccine Made From Embryonated Duck Eggs

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THE prevention of human rabies is of great concern to physicians treating animal bites. The development of rapid immunity to rabies virus is of prime importance.[1, 2] Unfortunately, one must face the possible occurrence of allergic encephalomyelitis as a result of brain antigen, possibly myelin, contained in present-day vaccine. The former problem has been partly resolved by the production of high potency vaccines and hyperimmune serum. Myelin, however, which has been implicated as a possible cause of encephalomyelitis,[3-5] is contained in all vaccines available at this time. This communication deals with further progress in the development of rabies virus vaccine of duck embryo origin. Vaccine of this origin contains little, if any, of the paralytic factor present in adult animal brain.

Two of us (H. M. P. and C. G. C.) have reported the development of fixed rabies virus of high titers in embryonated duck eggs.[6] Such a source has been found adequate for the production of potent antirabies vaccine.[7] It is almost devoid of encephalomyelitis-producing qualities as tested with Freund adjuvant in laboratory animals.[8] Jervis[9] has concluded that postrabic vaccine paralysis in human beings is comparable to that seen in guinea pigs injected with brain antigen and Freund adjuvant.

EXPERIMENTAL RESULTS

Duck embryo vaccine effectively immunizes mice in standard potency tests established by the National Institutes of Health.[10] It has also produced good virus-neutralizing antibody responses in two Cynomolgus monkeys given fourteen daily doses of vaccine. On days 0, 15, 20, and 30 the antibody titers[11] were 0-16-14-4 and 0-16-16-8 for these two monkeys. As a further laboratory trial of the vaccine, 43 dogs were given either duck embryo vaccine in 3 c.c. amounts or rabbit brain vaccine in 5 c.c. doses. In both groups, 80 per cent of the dogs had demonstrable antibody titers after 30 days. The commercial vaccine used on the dogs was assayed for potency by tests identical to those used to assay human antirabies vaccine. It is noteworthy that a smaller amount of duck embryo vaccine (3 c.c.) produced the same percentage of antibody responses as did a 66 per cent larger dose (5 c.c.) of commercial vaccine made from brain tissue.

From the Lilly Laboratory for Clinical Research, Indianapolis General Hospital, and the Lilly Research Laboratories.
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TABLE I. SUMMARY OF PATIENTS RECEIVING DUCK EMBRYO ANTIRABIES VACCINE

PATIENT NUMBER	AGE	BITTEN BY	NO. OF INNOC.	ERYTHEMA	INDURATION	TENDERNESS	ADENOPATHY	SYSTEMIC REACTIONS	ANTIBODY DETERMINATIONS
1	43	Dog	4	0	0	0	0	0	0
2	13	Dog	14	Slight	0	Moderate	0	0	3
3	69	Squirrel	14	Moderate	0	Moderate	0	0	3
4	7	Dog	14	0	0	Mild	0	0	2
5	7	Dog	9	Moderate	Moderate	Moderate	0	?	1
6	40	Dog	14	Slight	0	Moderate	0	0	3
7	10	Squirrel	14	Slight	0	Mild	0	0	3
8	39	Cat	14	Moderate	0	Moderate	0	0	3
9	21 mo.	Rat	14	0	0	Mild	0	0	0
10	17	Rat	14	Slight	0	Mild	0	0	3
11	6	Dog	14	0	0	Mild	Mild	0	0
12	4	Dog	4	0	0	Mild	0	0	0
13	4	Dog	11	Slight	0	Mild	0	0	0
14	18 mo.	Dog	6	0	0	Mild	0	0	0
15	45	Dog	14	Slight	0	Mild	0	0	3
16	45	Mouse	14	0	0	Mild	0	0	3
17	38	Dog	14	Moderate	0	Moderate	0	0	3
18	34	Monkey	14	Moderate	0	Moderate	0	0	3
19	23	Dog	14	0	0	Mild	Mild	0	2
20	5	Rat	14	0	0	Mild	0	0	0

CLINICAL TRIAL

The twenty patients selected for the initial clinical trial had minor bites and would not ordinarily have received commercial vaccine (Table I). Three of them voluntarily discontinued treatment (Nos. 1, 12, 14). One patient (No. 13), having contracted infectious diarrhea from a sibling who had the disease, was advised to stop the vaccine therapy. There was no reason to believe that the vaccine caused the diarrhea. Patient No. 5 developed local sensitivity to the injected material. In this instance induration and wheals developed around the injection sites, and the vaccine was stopped after the ninth day.

All patients complained of some tenderness at the injection sites. Mild to moderate erythema occurred in eleven of the patients, most often at the mid-period of the injection series (Table II.) Two subjects (Nos. 11, 19) developed

TABLE II. LOCAL ERYTHEMA AT INJECTION SITE—EACH X REPRESENTS ONE PATIENT-DAY OF ERYTHEMA. MOST ERYTHEMA OCCURRED IN THE MIDDLE PERIOD OF THERAPY

						X								
						X	X	X						
						X	X	X	X					
						X	X	X	X	X				
				X	X	X	X	X	X	X	X	X		
				X	X	X	X	X	X	X	X	X		
	X		X	X	X	X	X	X	X	X	X	X	X	X
Day	1	2	3	4	5	6	7	8	9	10	11	12	13	14

unilateral inguinal adenopathy. The enlargement was moderate and had disappeared at the end of 36 hours. No systemic reactions, i.e., headache, fever, or malaise, were noted. No abnormal neurological signs were found in any subject. None of the twenty patients receiving the vaccine have developed clinical symptoms of rabies during a period of from 1 to 4 months after the vaccine therapy.

DEVELOPMENT OF VIRUS-NEUTRALIZING ANTIBODIES

Serum for antibody determinations was obtained if possible on the first and last days of treatment and sixteen days after treatment had ended. Antibody concentration titers in Table III were based on standard bio-assay procedures and calculated by the method of Reed and Muench.[11] Three-hundreths milliliter of serum in varying dilutions and 100 LD_{50} of active rabies virus were administered intracerebrally to six mice per each serum dilution.

A first-day blood sample was not taken from five patients, of whom none gave a previous history of antirabies treatment. Schwab and her co-workers[12] did not find antibodies in 97 consecutive subjects who had never received rabies vaccine. It is presumed on this basis that the five previously mentioned individuals also had no antibody present on the first day. Four of these got an antibody response from the vaccine, while one did not. This one person (No. 6), a debilitated, chronic alcoholic, failed to develop antibody after 14 injections. In Patient No. 2 the immunologic response to the vaccine was minimal (less than a titer of 1:4). Patient No. 5 had a single blood sample withdrawn on the ninth day, and the antibody response was satisfactory.

TABLE III. VIRUS-NEUTRALIZING ANTIBODY RESPONSE OF HUMAN PATIENTS TO DUCK EMBRYO RABIES VACCINE

PATIENT	ANTIBODY TITERS (EXPRESSED AS RECIPROCAL OF THE SERUM DILUTION)		
	First Day	Fourteenth Day	Thirtieth Day
2	0*	†	†
3	0*	8	39
4	0*	7	11
5	0*	14 (day 9)	Not done
6	0*	0	0
7	0	16	164
8	0	27	19
10	0	11	128
15	0	4	†
16	0	12	11
17	50	2,048	2,440
18	0	109	Not done
19	0	12	45

Antibody titers are shown as the reciprocal of the serum dilution protecting 50 per cent of the mice as calculated by the methods of Reed and Muench.

Titers of 0 were probably 0 titers by analogy with another study but were not actually tested by us.

†Signifies an immunologic response in which some mice lived in the test but too few to give a 1:4 titer.

Seven patients, i.e., Nos. 7, 8, 10, 15, 16, 18, and 19, who showed no antibody by actual test of first-day blood samples, all got antibody responses from the vaccine.

Patient No. 17 gave a history of having received rabies vaccine five years previously. The pretreatment antibody titer, which was elevated, rose markedly by the fourteenth day, suggesting a "recall immunization." There was further elevation by the thirtieth day.

Thus, of thirteen patients tested, twelve had a demonstrable response to vaccine. This compares favorably, particularly in early appearance of antibody, with the series of Schwab, Fox, Conwell, and Robinson,[12] in which commercial rabbit brain vaccine was used. It may be safely assumed that very early appearance of antibody is a prime necessity in effective antirabies treatment.

CONCLUSIONS

The antigenicity of this vaccine when tested in experimental animals fulfilled the requisites of the National Institutes of Health mouse assay for potency. The lot of vaccine used in this study was one year old.

A clinical trial of the vaccine in twenty human subjects showed no untoward symptoms. Local reactions to the vaccine were very mild. Antibody tests done in thirteen of these subjects showed responses in twelve. A chronic alcoholic patient with delirium tremens did not get a response. Additional studies are in progress.

Duck egg albumin and chicken egg albumin are antigenically similar.[13] With the possibility in mind that a cross-sensitivity reaction theoretically might occur,[14, 15] persons sensitive to chicken egg albumin should not receive duck embryo vaccine.

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A STUDY OF THE INTERRELATIONSHIP OF THE ENERGY-YIELDING NUTRIENTS, BLOOD GLUCOSE LEVELS, AND SUBJECTIVE APPETITE IN MAN

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THE etiology of obesity is obviously complex, involving both physiologic and psychologic factors. The overt manifestations of the latter have tended to distract attention from the possibility that inherent defects in somatic mechanisms may play a role in the development of energy imbalance leading to active obesity. Thus, it has become important to define the physiology of appetite and hunger* and to try to relate it to the normal state of energy equilibrium as well as to seek evidence of its impairment at times when there is a positive or negative energy balance.

There is much evidence that the hypothalamus plays a vital part in the control of appetite,[1-4] and it has been identified as the site of a so-called appetite center. In order to function as such it is apparent that there must be a mechanism by which "information" as to recent food intake is available to the hypothalamus and sensitivity to some humoral factor, presumably either a specific nutrient or a product of intermediate metabolism, has been postulated. Mayer and his co-workers have suggested that the factor is not the "absolute" blood glucose level but the "available" blood glucose level as measured by the arteriovenous glucose difference, or delta (Δ) glucose, in accordance with the work of Foster[8] and Himsworth.[9] Work has been done in animals[5-7, 11, 12] and in man[10] to support this theory.

This paper presents the results of an experiment planned to test the satiety value of the three major energy-yielding nutrients, carbohydrate, fat, and protein, in a reducing situation, i.e., using isocaloric reducing diets and overweight subjects, and to attempt to relate changes in satiety value to the glucostatic theory of the regulation of food intake as proposed and elaborated by Mayer and his co-workers.[5-7, 10-12]

MATERIALS AND METHODS

1. *Subjects.*—The experimental group consisted of twelve adult males, eleven being graduate and undergraduate students, and one a faculty member. Relevant data for these are presented in Table I. The "Ideal Weight" was drawn from the tables published by

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*The terms "appetite" and "hunger" are used somewhat indiscriminately in this paper as their original context was in reports made by twelve experimental subjects whose interpretation of these two terms could be expected to differ. In general, "hunger" was used to express a more unpleasant and advanced "urge to eat" than "appetite," which represented a comparatively mild and almost pleasurable anticipatory sensation prior to meals. "Appetite," if unsatisfied, led to "hunger."

the Metropolitan Life Insurance Company, applying height, weight, and frame size. An index of the latter was calculated by a method devised by Showacre,[13] which is dependent upon the ratio of internal chest diameter, as measured radiographically, to height. The subjects varied from 10.4 per cent to 47.5 per cent above their ideal weights, and with one exception all were at least 20 pounds overweight. The health records of all subjects were examined to ensure that there was no evidence of relevant metabolic disorders, and a simple two-hour glucose tolerance test was carried out to eliminate the possibility of diabetes or prediabetes.

TABLE I. DATA ON EXERIMENTAL SUBJECTS

SUBJECTS	AGE	HEIGHT WITH SHOES (INCHES)	WEIGHT AS NORMALLY DRESSED (LBS.)	IDEAL WEIGHT	PER CENT ABOVE IDEAL WEIGHT
A	20	76.0	233.25	187	24.7
B	19	70.5	192.25	158	21.7
C	26	69.0	185.5	157	18.2
D	24	67.0	194.25	162	20.0
E	19	75.0	224.75	171	31.1
F	27	72.5	220.5	174	26.7
G	20	69.0	181.5	158	14.9
H	21	72.0	227.5	185	23.0
J	22	70.5	197.5	177	11.6
K	19	69.0	188.75	155	21.8
L	28	71.0	183.25	166	10.4
M	26	70.5	236.0	160	47.5

2. *Plan.*—The period of the main experiment was nine weeks, divided into three three-week phases. An additional phase of one week, using a different group of subjects, is reported separately later. During the three phases the experimental group ate all meals at a diet table under skilled supervision. Occasional meals away from the table were permitted during the first part of each phase, but the subject was asked to conform to the general dietary pattern and to report details of food eaten elsewhere. The subjects were asked to take no between-meal snacks, and the degree of cooperation was high. However, they were asked to report any lapses without fear of recrimination. These were recorded and subsequent scrutiny has shown that their over-all effect would be negligible. Throughout the experimental period diaries were kept by the subjects in which they recorded subjective sensations allied to appetite. These refer largely to degrees of between-meals "hunger" on arbitrary scales, but have enabled some estimate to be made of the relative satiety value of the diets used.

At the end of each phase, which represented a period of adaptation to a particular dietary intake, the subjects were confined to a metabolic unit for twenty-four hours, during which time venous and capillary blood samples were obtained for the determination of total reducing substances (subsequently the term "blood glucose level" will be used to indicate the results of these determinations since this is the widely accepted interpretation of the significance of "blood total reducing substances" although the terms are not, of course, synonymous). The first blood sample was taken before breakfast following a controlled overnight fasting period, and further samples were taken at eleven subsequent times throughout the day until 3½ hours after supper. The relationship of these times to meals was constant for all phases as follows: 60, 105, 165, and 225 minutes after breakfast; 75, 135, 195, and 255 minutes after lunch; 90, 150, and 210 minutes after supper. The meals eaten during the days on which blood samples were taken conformed to the diet schedule in force during the preceding three weeks. At the first eight sampling times paired venous and capillary samples were taken for the determination of the capillary-venous glucose difference. This has been found to be virtually identical with the arterio-venous glucose differences or Δ glucose.[14] The last four samples were capillary only. During the period in the metabolic unit physical activity was restricted to movement among the rooms in use, which were adjacent, and the subjects were virtually at rest.

TABLE II. DETAILS OF DIETS BY EXPERIMENTAL PHASES

PHASE	CALORIES	PRESCRIPTION (GRAMS)			PER CENT CALORIES		
		PRO.	FAT	CHO	PRO.	FAT	CHO
2	1,800	115.7	102.8	103.0	25.7	51.4	22.9
3	1,800	115.7	50.0	222.0	25.7	25.0	49.7
4 A	1,800	35.0	102.8	183.7	7.8	51.4	40.6
4 B	1,800	58.0	102.8	160.6	12.9	51.4	35.7
5	3,000	100.0	115.0	388.0	13.5	35.0	51.5

TABLE III. DIVISION OF NUTRIENTS BETWEEN MEALS (CONSTANT FOR EACH PHASE)

MEAL	CALORIES	PROTEIN	FAT	CARBOHYDRATE
Breakfast (approx.)	1/4	1/5	1/4	1/3
Lunch (approx.)	1/3	2/5	1/3	1/4
Dinner (approx.)	5/12	2/5	5/12	5/12

Diets: Details of the diets used in each phase are given in Tables II and III. All reducing diets were isocaloric at a level of 1,800 calories per day. The Phase 2 diet might be characterized as high in protein and fat and low in carbohydrate, whereas in Phase 3 the fat content was reduced by one half. During Phase 4 the experimental group was split into two subgroups, each of six persons. Group A received slightly less than one-third of the protein contained in the preceding diets and Group B one-half, the fat content being that used in Phase 2.

For ease of identification the diets used will subsequently be referred to as the low-carbohydrate diet (2), low-fat diet (3), low-protein diet (4a), moderate-protein diet (4b) and the 3,000 calorie diet (5), although it is stressed that the word "low" is used in these connotations in a purely relative sense. In all reducing diets the carbohydrate content was varied to maintain the over-all calorie intake at 1,800 daily. Thus when comparing any two diets there is always one constant component, fat or protein, and there are always two variable components, one of which is carbohydrate. Neither calories nor carbohydrate, fat, and protein components of the diets were divided equally among the three meals, the division being made to approximate that found in a normal regime. However, the relative proportions at each meal were kept constant throughout all phases.

Additional phase using 3,000 calorie diet: As an extension to the main experiment, an additional phase was added in which a 3,000 calorie diet was used. This phase lasted for one week, at the end of which blood samples were obtained as previously. The prescription for the diet was carbohydrate, 388 Gm.; fat, 115 Gm.; protein, 100 Gm. Because many of the original subjects were no longer overweight at the end of the main project, a new group of twelve subjects was recruited, an attempt being made to produce matched pairs with the first group with regard to "Ideal Weight" and percentage overweight. In fact, eight of the first group were closely matched, the other four subjects of the second group being lighter in weight than their counterparts but still at least 20 pounds above "Ideal Weight." The mean height and weight for the second group was 71 inches and 202 pounds, compared to 71 inches and 211 pounds for the first group.

3. *Methods.*—The blood glucose levels were determined by a modification of the Folin-Malmros method, as adapted by Horvath and Knehr.[15] It was found that more consistent results were obtained by using 1 ml. of potassium ferricyanide solution instead of the 2 ml. used in the original method; Duponal ferric iron was used instead of gum ghatti ferric iron in later determinations because of its easier preparation.

Venous samples were drawn by syringe from an antecubital vein, transferred immediately to a clean, dry test tube, and 0.1 ml. was pipetted into 10 ml. of tungstate solution.

The capillary samples were drawn into an 0.1 ml. pipette and thence into tungstate solution. This was then centrifuged at 3,000 r.p.m. for 10 minutes. Four milliliters of filtrate were pipetted into a tube graduated at 25 ml., 1 ml. of potassium ferricyanide and 1 ml. of sodium carbonate solution were added, and the tubes were placed in the boiling water bath for 20 minutes and cooled in running water for 3 minutes. Five milliliters of the ferric iron solution was added and the tubes were allowed to stand 5 minutes and were diluted to the mark. After mixing, the tubes stood 10 minutes before reading.

The standards (equivalent to 100 mg. per cent and one other level, either 50 mg. or 150 mg. per cent) were run in triplicate simultaneously with the samples.

The 100 mg. standard was set at 0.300 density on the Junior Coleman at wave length 520Å and the density of the unknowns was read against this standard as the point of reference.

RESULTS

1. *Satiety Value of Diets.*—Examination of the day-to-day diaries kept by the subjects and of an over-all appraisal written by them at the end of the project has made possible a comparison of the satiety values of the three main types of reducing diets used. This is shown in Table IV.

TABLE IV. SUBJECTIVE RESPONSE TO REDUCING DIETS

DIET	RANKED CHOICE OF DIETS FOR SATIETY VALUE		
	FIRST	SECOND	THIRD
2 (Low-CHO)	5	3	0
3 (Low-fat)	2	4	2
4 (Low-protein)	1	1	6

NOTE: 2 subjects ranked all 3 diets equal.
2 subjects made no adequate assessments.

Of the twelve subjects, two made no adequate assessments and two ranked all three diets equal in satiety value. The distribution of opinion among the other eight subjects ranks the low-carbohydrate diet the most "satiating" and the low-protein diets the least, with the low-fat diet holding an intermediate position. In general the difference between the diet low in carbohydrate (2) and that low in fat (3) was not great. However, comments made about the satiety value of the low-protein diets (4A and B), at both levels of protein intake used, were stronger. Four individuals were particularly incisive, complaining, in various terms, of being continually hungry.

2. *Blood Glucose Response.*—

a. *In capillary blood:* The mean capillary blood glucose levels for all diets are shown in Fig. 1. The fasting blood glucose levels vary in the range of 82 to 92 mg. per 100 ml., the maximum difference not being statistically significant at the 5 per cent level of probability. Breakfast produced a rise in blood glucose level ranging from 13 to 32 per cent of the fasting level at one hour after the commencement of the meal. The subsequent fall reaches fasting level in only one instance before the midday meal. The rise in blood glucose after lunch appears to be slightly higher than that after breakfast, but in fact no comparison is possible since there is a 15-minute difference in

the elapsed time between eating the meal and taking the blood sample. This underlines the very important fact that the points plotted as peaks and troughs on the blood glucose curves are not necessarily true maximum and minimum values since blood samples were taken only at relatively long intervals (approximately every hour). Any small difference among diets in the rate of

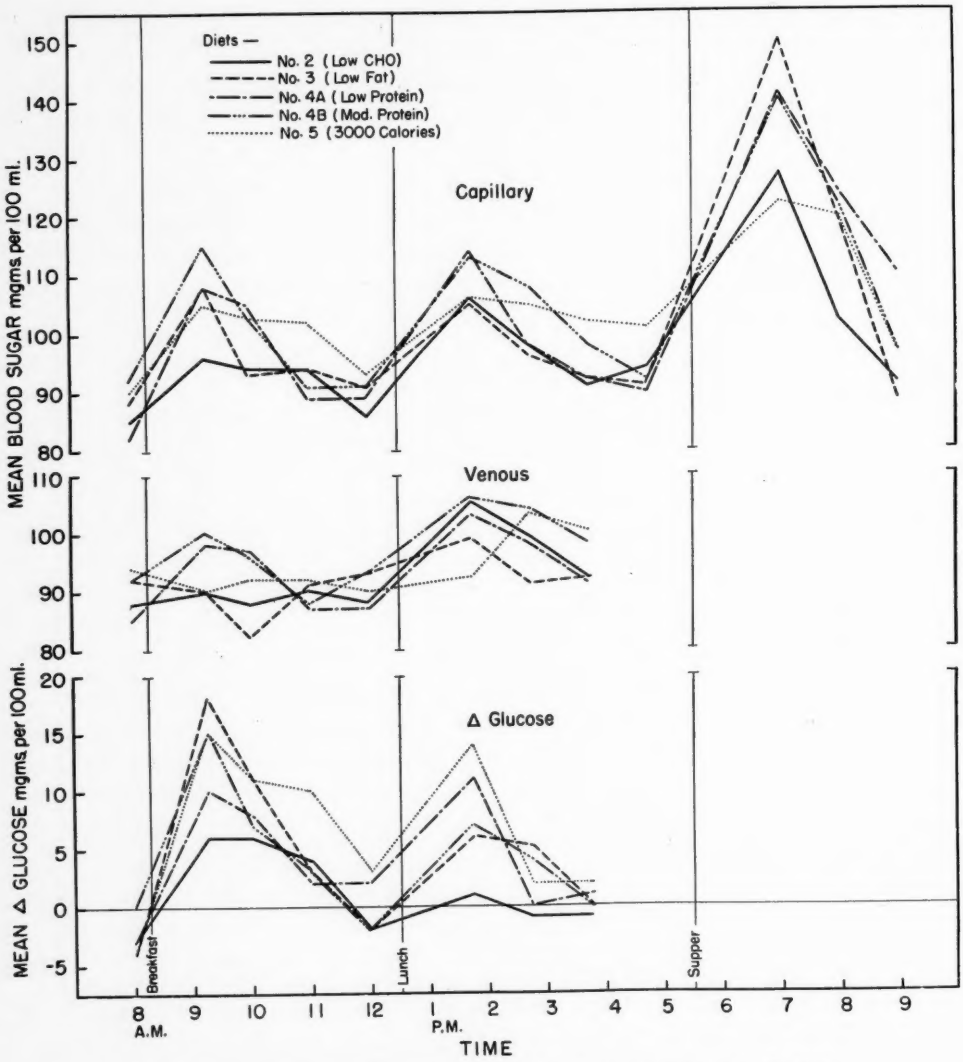

Fig. 1.—Mean capillary, venous and △ glucose levels on all diets.

development of the maximum postprandial blood glucose level would not be detected. Since the blood glucose level is being followed throughout a prolonged period, however, and the time intervals between blood samples are being held constant for all diets, the data are valid for comparisons among diets, although not for comparisons among meals. The rise in capillary blood

glucose levels after supper is strikingly greater than that following the preceding meals, and this is a constant observation for all diets. This effect is probably due mainly to the fact that supper had the highest calorie component of the three meals and also the greatest proportional carbohydrate component (Table III).

An analysis of variance for capillary, venous, and Δ glucose levels on all diets is shown in Table V. This shows the variation of capillary blood glucose levels among diets to be significant at the 5 per cent probability level. There is no apparent consistent relationship, however, between the ranked means of sums and any single dietary component. Thus, whereas the diet lowest in over-all carbohydrate content has the lowest ranked mean capillary blood glucose level, the diet with the second ranked mean capillary blood glucose level has the highest over-all carbohydrate content.

TABLE V. ANALYSES OF CAPILLARY, VENOUS, AND Δ GLUCOSE LEVELS

	SOURCE	D.F.	SUM OF SQUARES	MEAN SQUARE	F
1. Capillary (12 times; 1 missing observation)	Among diets	4	47,585.6	11,896.4	3.01*
	Among subjects within diets	42	166,119.3	3,955.2	
2. Venous (8 times; 2 missing observations)	Among diets	4	7,342.9	1,835.7	<1
	Among subjects within diets	41	91,528.3	2,232.1	
3. Δ Glucose (+30) Δ + 30 = C-V + 30 (8 times; 2 missing observations)	Among diets	4	11,481.7	2,870.4	5.76†
	Among subjects within diets	41	20,428.2	498.2	

Ranked Means							
	Capillary	Diets	2	3	4A	5	4B
		Means of Sums	1,168.1	1,220.3	1,231.8	1,243.6	1,256.0
	Venous	Diets	3	4A	2	5	4B
		Means of Sums	729.2	735.3	741.5	749.5	769.3
	Δ Glucose	Diets	2	4A	4B	3	5
		Means of Sums	11.1	30.8	37.5	39.0	53.9

*Significant at 5 per cent probability level. †Significant at 1 per cent probability level.
Mean of sum = sum of capillary, venous, or Δ glucose levels for all subjects at all sampling times on a given diet, divided by the number of subjects.

It is of interest to note that on a 3,000 calorie intake the fasting capillary blood glucose level was of the same order as those which occurred with the 1,800 calorie diets and the postprandial rise is, in all instances, exceeded by that of one or more of the reducing diets. This is a clear manifestation of increased blood glucose tolerance with a higher calorie intake. The one apparent difference between the two levels of calorie intake appears to be in the duration of the elevation of capillary blood glucose level after breakfast and after lunch, the higher intake being associated with a more prolonged elevation, as one would expect.

b. *In venous blood:* The mean venous blood glucose levels for all diets are shown in Fig. 1. The variation between absorptive and postabsorptive

states is considerably less than that occurring in the capillary blood, so that the curves are flatter. On two diets a fall rather than a rise occurred in the mean venous blood glucose level after eating breakfast and this was seen to happen in 14 of the 48 individual venous blood glucose curves obtained (29 per cent), being a constant finding for two of the individual subjects on all diets. In these cases peripheral utilization of glucose apparently outstrips the increased supply during the absorptive period.

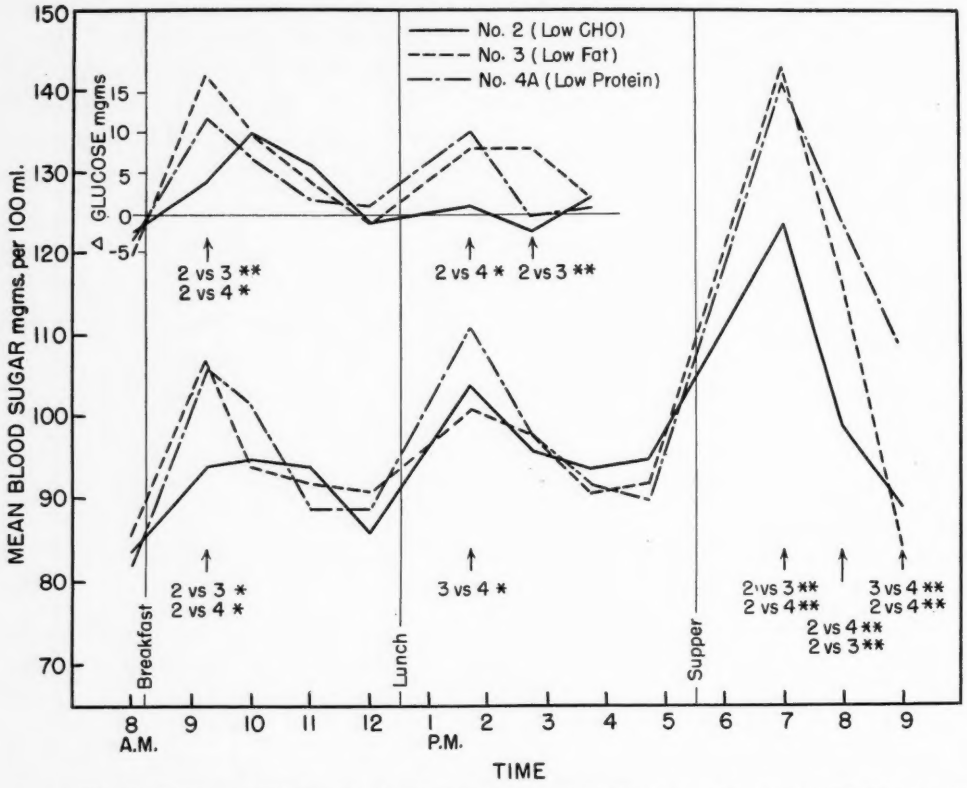

Fig. 2.—Mean capillary and △ glucose levels on Diets 2, 3, and 4A (6 subjects, A, B, C, E, J, K). Indication is given below the curves of the statistical significance of differences in capillary or △ glucose levels at the various sampling times. Thus 2 vs. 3** means that the difference in mean glucose levels for Diets 2 and 3 at the time indicated was significant at the 1 per cent probability level. Where no indication is given, differences between glucose levels were not significant at the 5 per cent probability level. Statistical significance tested by Duncan's procedure.

Statistically the differences in mean venous blood glucose levels among diets were not significant at the 5 per cent probability level, but the ranked means of sums are in the same order as the capillary levels with the exception of Diet 2, which had a higher rank than Diets 3 and 4A for venous blood. No meaningful relationship with individual dietary components is apparent.

c. *Capillary-venous difference or Δ glucose:* The mean Δ glucose values for all diets are shown in Fig. 1. They demonstrate a consistent rise and fall coincident with the absorptive and postabsorptive states, although the differences among diets are considerable, being significant at the 1 per cent probability level (Table V).

The height of the rise in mean Δ glucose after meals is not consistently related to any single dietary component, but the 3,000 calorie diet (5) appears to maintain a higher level, especially about 3 hours after eating, and it has the highest ranked mean. Conversely, the diet low in carbohydrate (2) has a comparatively low mean and is ranked last among the five diets. It is noticeable that on this diet the rise in mean Δ glucose after lunch was comparatively slight; this was not associated with any decrease in its satiety.

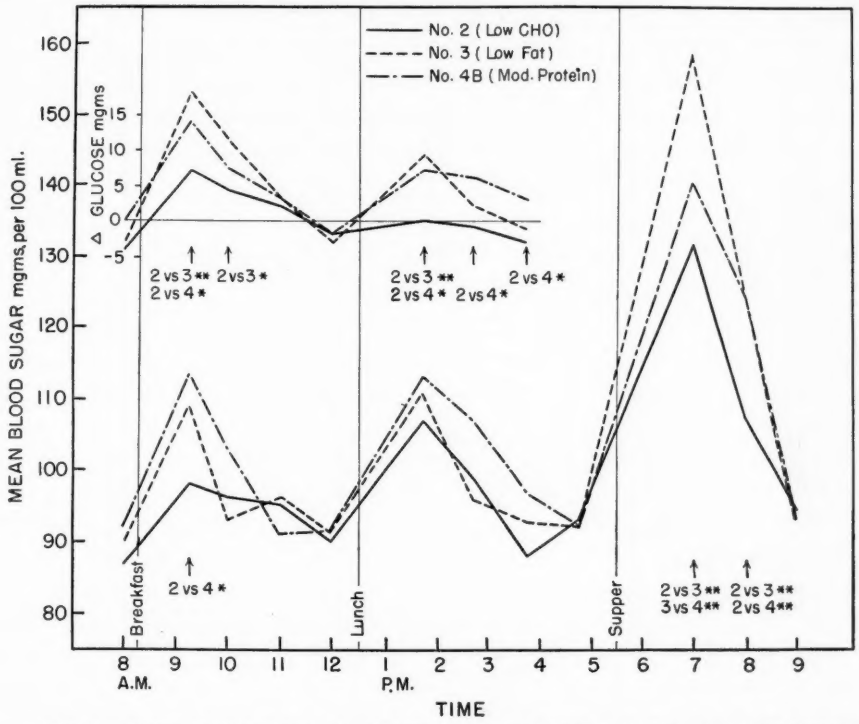

Fig. 3.—Mean capillary and △ glucose levels on Diets 2, 3, and 4B (6 subjects, D, F, G, H, L, M). Indication is given below the curves of the statistical significance of differences in capillary or △ glucose levels at the various sampling times. Thus 2 vs. 3** means that the difference in mean glucose levels for Diets 2 and 3 at the time indicated was significant at the 1 per cent probability level. Where no indication is given, differences between glucose levels were not significant at the 5 per cent probability level. Statistical significance tested by Duncan's procedure.

Examination of the glucose responses of individuals revealed considerable variation of pattern. In some instances the capillary glucose level one hour after eating would be almost back to fasting level but this finding was commonly associated with a fall of the venous glucose below fasting level, thus establishing a rise in the Δ glucose. Many individual instances of low or negative Δ glucose levels unassociated with any subjective sensations of appetite or hunger were noted. The converse, hunger coinciding with a substantial Δ glucose level, occurred in two individuals on the low-protein regime.

Comparative aspects of results: Comparisons of the effects of Diets 2, 3 and 4A and of 2, 3, and 4B were made separately for subjects A, B, C, E, J,

K, and D, F, G, H, L, M, respectively. It was decided that this partitioning of the data led to reasonably efficient statistical procedures for comparing differences in blood glucose levels at individual times throughout the day. The results for capillary and Δ glucose values are presented graphically in Figs. 2 and 3. Significant differences in capillary and Δ glucose levels appear mainly in samples taken at the height of the absorptive period and, in general, become significant at a 1 per cent probability level only after supper; this being the largest meal of the day, it would be expected to exaggerate any differences due to the diets themselves.

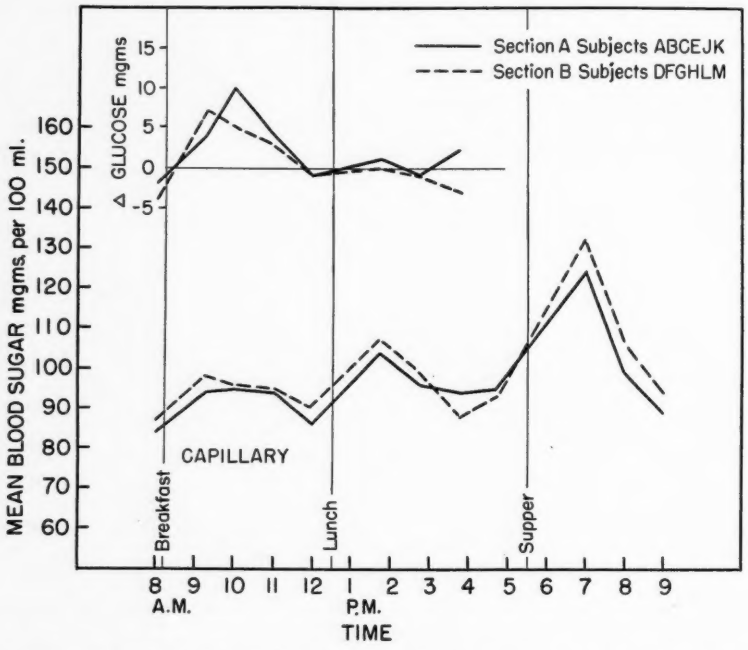

Fig. 4.—Comparison of mean capillary and △ glucose levels for two subgroups of six subjects on a low-carbohydrate diet (2).

When the mean capillary and Δ glucose curves for the two subgroups on Diet 2 are superimposed, as in Fig. 4, it becomes apparent that there was a remarkable homogeneity of response between the subgroups, and this was also true for Diet 3. This observation encourages the belief that the mean glucose responses from which conclusions have been drawn are typical of those which would be expected from the population sampled. Variation among subjects was, however, considerably greater for subgroup A than for subgroup B. Direct statistical comparison of the blood glucose responses to Diets 4A and 4B is, therefore, avoided because effects due to groups and diets would be indistinguishable.

3. *Loss of Weight by Subjects.*—The loss of weight by the subjects in the experimental group was entirely incidental to the main purposes of the study except inasmuch as it provided evidence of close adherence to the dietary

regime. A daily requirement of about 3,200 calories would seem to be reasonable for the subjects who formed the experimental group, having regard to their age and activity levels. The 1,800 calorie diets fed, therefore, represented a daily deficit of 1,400 calories which, on theoretical grounds, would lead to a weight loss of 2.8 pounds per week. In fact, the mean weight lost during the nine weeks on 1,800 calories a day was 24.7 pounds, or 2.6 pounds per week.

DISCUSSION

Hunger and appetite are necessarily subjective and unquantifiable in man and hence the assessment of satiety values presented in Table IV must be regarded as only a crude indication of any differences among the diets used. Nevertheless it does seem to provide reasonable evidence that the protein component of a diet is more important than either fat or carbohydrate components in satisfying appetite, at least in the critical situation of a caloric deficit. This observation is in agreement with the findings of Thorn and associates[16] and Orent-Keiles and Hallman.[17] These investigators associated the satiety effect of the protein content of the breakfast meal with a delay in the return of blood glucose to fasting levels. On general grounds, accumulated experimental evidence is against the controlling humoral factor being the absolute blood glucose level as opposed to the available blood glucose level (as evidenced by the Δ glucose). The fact that a diabetic may be normally hungry or even hyperphagic would also require explanation. Furthermore neither of these investigators used statistical methods to evaluate differences in the blood glucose levels resulting from different diets. Bryant and her co-workers,[18] using normal subjects in two age groups, found that the postprandial rise in blood glucose level following breakfast was prolonged by increasing the amount of protein in the meal and that protein of plant origin was as effective as animal protein in producing this effect. While these results were evaluated statistically, the method used was inappropriate, to say the least, and examination of the data presented reveals that the actual difference in mean blood glucose levels to which statistical significance was attached was only 2 mg. per 100 ml. In general, therefore, attempts to explain the phenomenon of the apparent satiety effect of protein have been based on two assumptions: (1) that appetite is in some way regulated by changes in absolute blood glucose levels or in the Δ glucose level, (2) that the process of neoglucogenesis from protein sources would provide a more prolonged elevation of absolute peripheral blood glucose levels or of the Δ glucose levels than would result from the breakdown of a comparable amount of carbohydrate. Neither our results nor those of Haldi and Wynn[19] confirm either of these assumptions.

Examination of the capillary and Δ glucose curves for the four reducing diets used (Fig. 1) shows no consistent correlation of the height of the glucose response with any single dietary component. However, the low-carbohydrate diet (2), which had the highest satiety value, also shows the lowest ranked

mean values for both capillary blood glucose and Δ glucose. Conversely, the diets low in protein (4A and 4B) with a comparatively lower satiety value have the highest ranked mean value for capillary blood glucose, and the mean Δ glucose value exceeds that found with the low-carbohydrate diet by a significant amount. Examination of the data for subjects F, J, H, and M further emphasizes the lack of positive correlation between glucose response and satiety. These were four individuals whose hunger on the low-protein regime was most marked, but who found that in general the low-carbohydrate diet left them with only normal appetite before meals. As can be seen from Fig. 5, the glucose responses were appreciably greater on the diet of low satiety value at all times when, in the over-all study, differences among diets were found to be of statistical significance; that is, during the absorptive period.

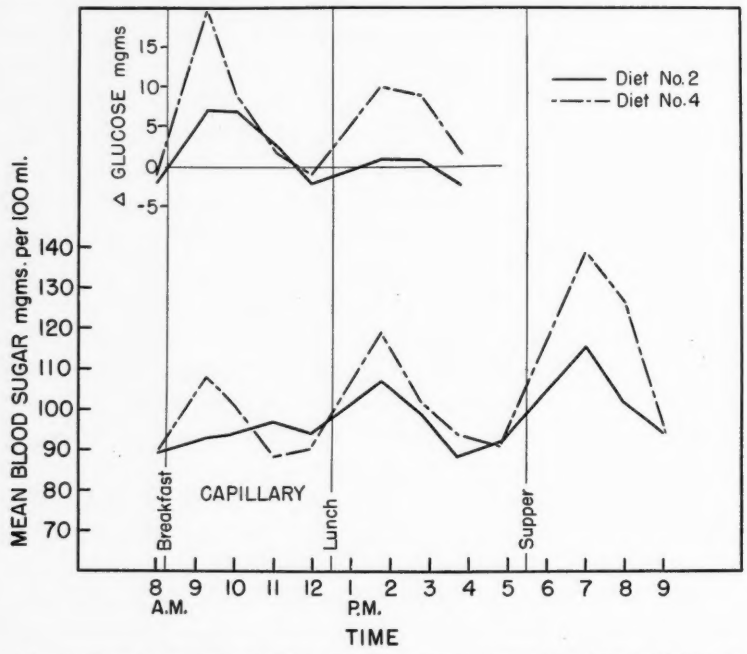

Fig. 5.—Comparison of mean capillary and △ glucose levels for four individuals (F, J, H, M) on a low-carbohydrate diet (2) and low- or moderate-protein diets (4A or B).

It is to be expected that psychologic factors will play a part in determining the satiety value of any diet. The common sources of first-class protein such as meat, eggs, and milk occupy a prominent place in the daily dietary of a majority of the population of the western hemisphere, and the sudden elimination of a substantial proportion of such foods from the diet would be likely to lead to a lack of "satisfaction" at the time meals were eaten, if not to the appearance early in the postprandial period of subjective sensations which might be indistinguishable from hunger or appetite caused by any physiologic mechanism. It seems unlikely that psychologic factors alone could cause the quite marked loss of well-being and efficiency as well as the prolonged periods of between-meals hunger reported by several of the experi-

mental group on the low-protein regime, especially as the group was originally carefully selected with the aid of a psychologist to include only emotionally stable subjects likely to cooperate fully in the project. It is conceded, however, that the possibility of the so-called satiety value of protein being entirely due to psychologic factors has not been scientifically disproved. If it is assumed that there are physiologic factors involved, then the implication is that the special contribution made to the satiety value of a diet by its protein component is due to the direct or indirect effects of some protein metabolite or perhaps to the process of protein metabolism itself as evidenced, for example, by its specific dynamic action. Strominger and Brobeck[3] have suggested this as a possible factor. The present experiment has demonstrated that such effects are not associated with the changes in absolute blood glucose or in Δ glucose levels which should occur if the regulation of appetite depended upon a purely "glucostatic" mechanism. On theoretical grounds alone protein would not be expected to contribute as much to the blood glucose as an equivalent weight of carbohydrate, nor does it appear to prolong the postprandial elevation of blood glucose, as had been postulated (see Fig. 1).

Mayer and his co-workers[5-7, 10-12] have demonstrated many instances of changes in appetite associated with hyperglycemia and hypoglycemia, and the attractiveness of the glucostatic theory, as such, is undeniable, providing, as it does, a link between the immediate supply of nutrients and the hypothalamus and safeguarding directly the essential metabolite of the central nervous system. Changes in blood glucose levels are, however, associated with complex parallel changes throughout the metabolic pool, and it would seem reasonable to postulate that it may be some individual metabolite or, better still, the over-all balance between metabolites within this integrated system which provides the humoral mechanism controlling appetite through the mediation of the hypothalamic centers. Such a theory transfers the emphasis from blood glucose, which stands, so to speak, on the carbohydrate portal of the metabolic pool to the dynamic equilibrium existing on the "final common pathway" of metabolism, and would adequately explain Mayer's findings while allowing for differential factors compatible with the apparent superiority of protein in providing satiety.

SUMMARY AND CONCLUSIONS

Twelve overweight subjects were fed isocaloric reducing diets in which the proportions of carbohydrate, fat, and protein were varied. After a three-week adaptation period to each diet, capillary and venous blood samples were obtained from each subject throughout a full day during which normal meals were eaten from the diet.

It was found that there was a marked reduction in the satiety value of the diets containing the lower protein components.

The mean capillary, venous, and Δ glucose values resulting from the various diets are discussed and a statistical evaluation of their differences is made. The effect on the glucose curves of increasing the calories from 1,800 to 3,000 a day was tested using a matched experimental group.

No correlation was found between the capillary, venous, or the Δ glucose levels and the satiety values of the diets used. On the contrary, the diet having the greatest satiety value produced the lowest mean blood glucose and Δ glucose levels. Many individual instances were found of complete lack of hunger in the presence of negligible or even apparently negative Δ glucose levels and, conversely, in two individuals of marked hunger in the presence of substantial postive Δ glucose levels.

No confirmation was, therefore, obtained for the "glucostatic theory" of appetite regulation, but it is suggested that the humoral mechanism controlling the appetite may depend upon levels of individual metabolites or the over-all state of equilibrium on the "final common pathway" of metabolism.

We wish to express our thanks to Dr. L. A. Maynard for much helpful advice and criticism; to Drs. E. C. Showacre, R. G. Steel, and J. Summerskill for their respective assistance with radiologic, statistical, and psychologic aspects of the study; and to G. Fiala, B. Einset, J. Boss, L. Mendiola, and others for technical assistance.

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CLINICAL EXPERIENCE WITH INTRAVENOUS INFUSION OF EMULSIFIED FAT*

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IN RECENT years there has been a renewal of interest in the use of emulsified fat for intravenous nutrition.[1-3] In general these reports have been encouraging, but they have pointed out many obstacles to be overcome before wide application of this form of therapy can be considered. These concern stability, pyrogenicity, vasomotor and allergic reactions, as well as other problems associated with the introduction of colloidal material into the blood stream. The utilization of intravenous fat,[4-7] the methods by which it leaves the blood,[8, 9] and its effect upon hepatic function have been studied.[10] The present report concerns the results of 1,466 infusions of fat emulsions in 426 patients and represents the larger part of our clinical experience with fat emulsions during the period from September, 1950, to July, 1954.

The incidence of thermogenic responses to the infusion of emulsified fat has been one of the major difficulties that has confronted workers in this field. At the present time there is still uncertainty about the origin of these pyrogens and their mode of operation. Contamination of raw materials, hydrolytic products from oils and emulsifiers, hemolytic agents, nonspecific colloidal phenomena, and products of reactions between emulsifiers, oils, and proteins of blood and tissue have been considered as possible sources of thermogenic reactions to infusion of fat emulsions. There is little objective evidence to indicate which, if any, of these possible factors are important. Indeed, where improvements have been made they have been made on an empirical basis. When these clinical studies were begun it was believed that pyrogenicity resulted in large part from contamination of raw materials. Consequently many different sources of a given oil as well as different oils were used, and considerable effort was made to eliminate possible sources of contamination and to remove traces of contaminants by chemical and physical purification. This effort has been continued to date, using both natural and synthetic oils.

It has been necessary to evaluate numerous natural and synthetic emulsifying agents and triglycerides in the laboratory, and those which appeared promising were eventually tested in the clinic. For these reasons a large number of different batches of emulsions have been prepared, tested in animals, and finally

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applied clinically. During the period covered by this report more than 1,000 different experimental and clinical emulsions have been prepared, and of these, 151 have been used in patients.

The data presented are a summary of the clinical observations that have been made. The results of the study of pathologic material accumulated during the course of these studies will be presented elsewhere. Preclinical testing of the emulsions included infusion intravenously into three species of animals—rats, dogs, and rabbits—plus microscopic examination of the emulsion using the phase microscope. All emulsions were also tested for sterility. The details of these studies are given in a separate paper.[11] Emulsions which passed all preclinical tests were then used in patients. In using the emulsions in man, if any particular batch gave rise to a series of reactions it was discarded, and similarly if any individual patient reacted severely to administration of fat infusions he was usually given no further infusions.

A temperature rise of more than two degrees, a chill, or any obvious reaction has always been considered adequate reason for stopping an infusion. On the other hand, if a given patient tolerated the infusions well, he may have been infused daily for several weeks. For these reasons the data are weighted favorably. Using these methods we have found that approximately 100 infusions in at least 25 patients are required to gain an adequate evaluation of the clinical usefulness of any particular type of emulsion. Clinical testing of this sort has been and still is necessary to determine the final behavior of any particular emulsion or batch of emulsion, because to date it is impossible to predict the clinical result on the basis of laboratory and animal experimentation.

EXPERIMENTAL

The methods of manufacture, the standards that have been set, and the controls that accompany and precede each infusion are described in a separate paper.[11] Suffice it to mention here that the preparation and composition of the emulsion were similar to those previously described from this laboratory.[1, 7] Fat was used in concentrations from 10 to 15 per cent (w/v), phosphatide generally at 1 to 1.2 per cent (w/v), and a costabilizer, Demal-14, Emcol, or Pluronic, at 0.2 to 1.0 per cent (w/v). Sterile, pyrogen-free, isotonic dextrose solution was used as the aqueous phase. The fats used are given in the appropriate tables.

Because of the limited production and storage facilities, the emulsions were used within 2 months of their manufacture, but some have been used satisfactorily after a much longer time.

Patients.—Almost all the 426 patients in these studies were ill and in need of caloric supplementation. All but a few were being treated on the wards of the Massachusetts General Hospital, and these few were patients in other local hospitals. The group as a whole therefore constitutes a reasonable cross section of severely ill patients in a large general hospital. Almost every common type of disease that might be associated with acute or chronic starvation is represented, but the majority were suffering from gastrointestinal tract neoplastic disease or intra-abdominal infection. Of the total number of 426 patients who received one or more infusions, 83 were running a septic course. These febrile patients have been separated and the data collected on them analyzed separately, since in many instances it was impossible to evaluate pyrogen response. The tables given include only the data pertaining to patients who were nonfebrile, unless otherwise designated.

Collection of Data.—At each infusion the temperature, pulse, and blood pressure were recorded by research assistants participating in this study, once before the infusion of fat

began, every twenty minutes for the first hour, and at hourly intervals thereafter until the infusion was finished. In addition, any late rises in temperature apparent from the clinical chart were recorded. Subjective responses of the patients were recorded as well as any objective evidence of distress such as flushing, apprehension, and pain. The majority of the infusions given to adults were administered in quantities of 600 ml., although 1,200 ml. infusions were not uncommon. These quantities for infusion were arrived at simply because the bottles most satisfactory for autoclaving the finished emulsion were of 600 ml. capacity.

If any severe reactions occurred, such as chills or a temperature rise of two or more degrees, the infusion was terminated. The development of back pain (see later in the article) was usually not considered sufficient reason to abandon the infusion, for after a twenty-minute rest period it was possible in most instances to continue. The rate of administration was usually 20 drops (1 ml.) per minute during the first twenty minutes, 60 drops (3 ml.) per minute thereafter. Slower rates of administration were tried on occasion, but the results were not sufficiently different to allow a definite conclusion, although it is our impression that a slightly lower incidence of all types of reactions would occur under these circumstances.

Most of the reactions occurring during the infusion of emulsified fat have fallen into fairly narrow patterns. These have been categorized as pyrogen responses, chills, colloid phenomena, vasomotor changes, and miscellaneous reactions. They deserve individual comment.

Pyrogen response is the most frequently observed untoward reaction. Most such reactions are similar to the febrile responses usually associated with pyrogens, namely, a rather sharp rise in body temperature, often associated with chill. The temperature falls within one or two hours and does not rise again. We have no information on what would happen if infusions were continued despite a rise in temperature above 2° F. The temperature often rises higher after the infusion is stopped. In a few instances there have been temperature rises several hours after completion of the infusions. These have been mild but quite definite.

Chills usually occur in association with thermogenic responses. However, a mild form of chill or chilly sensation without temperature elevation has been observed.

Colloid Phenomena.—Under this heading are included a number of reactions which we believe are related to one another and due to the introduction of colloids into the blood stream. The reaction occurs infrequently and in most instances only during the first infusion. In its severe form the first few milliliters of emulsion (severe reactions have occurred with less than 1 ml. of emulsion) lead to the development in rapid order of flushing and warmth of the face and neck, apprehension, sensation of constriction or oppression in the chest, cyanosis, and severe pain in the back, sometimes from the base of the skull to the sacrum. The pain may radiate down the legs, into the groin or flanks. Fortunately this train of events has occurred only a few times. More often the patients have experienced only a back pain, and the severity of this is variable. Symptoms can occur singly or in any combination. With such symptoms the infusions have always been stopped temporarily. They lessen in 2 to 3 minutes after stopping the infusion and in 5 to 10 minutes disappear altogether. In most instances the infusions can be resumed after 20 minutes or the next day without any untoward reactions. Only four patients have had reactions of this sort on more than one occasion. No apparent ill effects have

TABLE I. REACTIONS TO EMULSIONS CONTAINING DIFFERENT OILS

OIL	NUMBER PATIENTS	NUMBER INFUSIONS	NUMBER INFUSIONS PER PATIENT	DEGREES RISE FAHRENHEIT					CHILLS	COLLOID REACTION
				0-1°	1.1°-2.0°	2.1°-3.0°	3.1°-4.0°	4.1°+		
				PER CENT OF TOTAL NUMBER OF INFUSIONS						
Coconut	74	261	3.5	86	5	6	1	1	5	3
Olive	187	758	4.1	82	7	5	4	2	6	1
Coconut + olive	33	88	2.7	85	9	3	1	1	4	1
Peanut	9	18	2.0	83	6	6	0	6	17	0
Cottonseed	132	252	1.9	91	6	2	1	0	4	2
Synthetic triolein	23	39	1.7	79	15	5	0	0	7	0
Purified olive	21	50	2.4	76	12	8	4	0	8	4

Cottonseed oil and synthetic triolein appear superior.

followed such reactions. During the acute phase of the reaction the systolic blood pressure is mildly elevated. The urine remains clear. No evidence of hemolysis has been found. Clinical observation suggests that these reactions are similar to reactions to blood transfusion accompanied by back and flank pain. This reaction is similar to, if not identical with, the "early" type of reaction which has been reported as a complication of intravenous administration of colloidal iron. Nissim[12] has recently collected the available data pertaining to these reactions. At one time or another during the past 4 years we have observed most of the symptoms and signs listed in his review. We feel quite certain that embolization could not account for these reactions. The onset is too abrupt and the quantity of emulsion is usually too small to occlude many capillaries. The fact that infusions of large amounts of fat can be uneventfully carried out a short time later also indicates that embolization is not the cause of these reactions. Visceral ischemia, however, might be involved secondarily due to an unusual vascular response caused by the sudden introduction of one mixture of colloids into a second, namely, the blood. This statement is based upon the similarity of responses to the injection of colloidal iron and of fat emulsion, also upon unpublished observations[13] that apparently identical reactions occur after the intravenous injection of inulin in colloidal suspension rather than complete solution. These same facts suggest the unlikelihood that materials present in noncolloidal form are responsible. Antihistaminics given with the fat infusion have not lessened the incidence of these reactions. Allergy has been suggested, but the evidence is not consistent with any known form of allergic or hypersensitivity reaction. Patients with inflammatory lesions seem more susceptible to these reactions.

Vasomotor changes have occurred commonly. These involve a rise in systolic blood pressure, little or no change in diastolic blood pressure, and moderate elevations of pulse rate. The latter sometimes occur in association with other reactions. These changes appear to be dependent upon the emulsifying agents that have been used, as will be illustrated later. They are quickly reversible and have always subsided on slowing the rate of infusion.

Miscellaneous reactions include a few responses that have been considered to be allergic (skin rash in three patients; asthma, one patient), nausea, vomiting, anorexia, and headache. In the case of nausea and vomiting it was often difficult to tell whether they resulted from the fat emulsion or the patient's disease. The incidence of these reactions is low, less than 1 per cent.

RESULTS

Different Oils.—In Table I are presented data concerning the incidence of chills, pyrogen, and colloid reactions as related to the oil employed. Superficially the data appear quite similar, but very definite improvement from a pyrogen standpoint resulted from the use of cottonseed oil and synthetic triolein. Less than a one-degree rise in temperature was observed in 91 per cent of the infusions with emulsions of cottonseed oil. From a clinical point of view we have felt that any febrile response of less than two degrees (100.6° F.), when not accompanied by a chill, was tolerable. Using this criterion 97 per cent of all

cottonseed infusions were satisfactory and 94 per cent of all infusions of synthetic triolein emulsions. It is also notable that among the last 341 infusions with emulsions of cottonseed oil, synthetic triolein, and purified olive oil no patient had a temperature rise of more than four degrees, i.e., none over 102.6° F. Emulsions of coconut oil and coconut plus olive oil gave results almost as good. One of the most significant results shown in Table I is the successful infusion of wholly synthetic triolein emulsions. Although the number of infusions is small, the number of patients is substantial, and the figures listed are a fair representation of the probable results of a larger number of infusions. These results indicate that the oleic acid content of the oil triglycerides is probably not the determining factor in pyrogenicity.

TABLE II. COMPARISON OF DIFFERENT BATCHES OF COTTONSEED OIL EMULSION

EMULSION NUMBER	NUMBER PATIENTS	NUMBER INFUSIONS	DEGREES RISE FAHRENHEIT					CHILLS	COLLOID REACTION
			0-1°	1.1°-2.0°	2.1°-3.0°	3.1°-4.0°	4.1+		
			PER CENT OF TOTAL NUMBER OF INFUSIONS						
1	10	34	91	9	0	0	0	6	0
2	27	35	97	3	0	0	0	0	11
3	33	65	94	6	0	0	0	0	0
4	35	61	85	10	2	3	0	8	3
5	8	24	92	0	8	0	0	4	0
6	8	12	92	0	8	0	0	8	0
7	7	16	87	6	6	0	0	0	0

It is concluded that there is variation in the result obtained with different batches.

In Table II the results of infusion of cottonseed emulsions are broken down by individual batches. It can be seen that there is still variation among batches. Unfortunately we have been unable to find the causes of the variable results among different batches. These may result from insufficient control of manufacture of small experimental lots, or from testing on relatively small groups of patients.

Table III summarizes the data pertaining to rise in systolic blood pressure resulting from the infusion of emulsions of different oils. These results were essentially the same for all oils except peanut, and additional data will be required for evaluation of the latter.

As regards the colloid reaction and chills (Tables I and II), it seems that little if any progress has been made toward elimination or even reduction of the incidence of these undesirable effects. The group of patients who received synthetic triolein emulsions is small, but further work with this triglyceride is in progress.

Concentration of Oil.—In Tables IV and V the data relative to different concentrations of oil are presented. This comparison is made regardless of the emulsifying agents or their concentration or the type of oil. In general the 15 per cent emulsions contained slightly larger amounts of emulsifiers. It is believed that the differences are not sufficiently great to indicate that variations of oil concentration between ten and fifteen per cent have any effect upon the incidence of pyrogen reactions. The data in Table V show significant difference in the incidence of systolic blood pressure rise between 10 per cent emulsions on the one hand and 12.5 and 15 per cent emulsions on the other. It is possible

that with higher concentrations of oil more phosphatide may be distributed at the oil-water interface because the total area of this interface is expanded as the concentration is raised.

TABLE III. SYSTOLIC BLOOD PRESSURE RESPONSE IN PATIENTS RECEIVING EMULSIONS OF DIFFERENT OILS

OIL	NUMBER PATIENTS	NUMBER INFUSIONS	MILLIMETERS OF MERCURY RISE					
			0-10	10-20	20-30	30-40	40-50	50+
			PER CENT OF TOTAL NUMBER OF INFUSIONS					
Coconut	72	356	31	24	20	13	5	8
Olive	170	903	34	23	18	11	8	7
Coconut + olive	30	90	37	12	21	12	11	7
Peanut	7	15	67	7	13	13	0	0
Cottonseed	91	216	25	12	22	20	10	11
Synthetic triolein	25	35	29	34	17	6	9	3
Purified olive	18	36	30	25	19	14	0	11

In this and the succeeding tables pertaining to blood pressure, the data collected on febrile patients are included. It was not feasible to collect blood pressure data on every patient—hence the discrepancies between the number of infusions included in the blood pressure tables and the other tables. It is concluded that the oil used is probably not a determining factor in causing vasopressor response and that this reaction is not related to pyrogen response..

TABLE IV. REACTIONS TO EMULSIONS OF DIFFERENT OIL CONCENTRATIONS

CONCENTRATION OIL (%)	NUMBER PATIENTS	NUMBER INFUSIONS	DEGREES RISE FAHRENHEIT					CHILLS	COLLOID REACTION
			0-1°	1.1°-2.0°	2.1°-3.0°	3.1°-4.0°	4.1+		
			PER CENT OF TOTAL NUMBER OF INFUSIONS						
10	251	669	85	6	5	3	1	5	1
12.5	67	216	88	4	4	1	2	6	0
15.0	104	359	84	8	4	2	1	6	1

It is concluded that variations in oil content between 10 and 15 per cent do not significantly alter the incidence of these reactions.

TABLE V. SYSTOLIC BLOOD PRESSURE RESPONSE IN PATIENTS RECEIVING EMULSIONS OF DIFFERENT OIL CONCENTRATIONS

OIL (%)	NUMBER PATIENTS	NUMBER INFUSIONS	MILLIMETERS OF MERCURY RISE					
			0-10	10-20	20-30	30-40	40-50	50+
			PER CENT OF TOTAL NUMBER OF INFUSIONS					
10	105	486	29	18	16	14	11	11
12.5	55	217	39	25	17	7	5	6
15	99	435	40	22	16	11	6	5

There appears to be a difference between emulsions containing 10 per cent oil and those containing 12.5 and 15 per cent oil. However, there are several other variables which detract from the significance of this difference, for example, the distribution of phosphatide between oil and water phases. These data are considered to be consistent with the concept that distribution of emulsifiers in a larger bulk of oil protects against higher vasopressor responses.

TABLE VI. THE EFFECT OF DIFFERENT CONCENTRATIONS OF PHOSPHATIDE

CONCENTRATION PHOSPHATIDE (%)	NUMBER PATIENTS	NUMBER INFUSIONS	DEGREES RISE FAHRENHEIT					CHILLS	COLLOID REACTION
			0-1°	1.1°-2.0°	2.1°-3.0°	3.1°-4.0°	4.1+		
			PER CENT OF TOTAL NUMBER OF INFUSIONS						
0.5	68	311	86	6	4	2	2	7	2
1.0	74	214	81	6	7	4	1	7	2
1.2	182	464	84	8	4	2	1	5	1
2.0	19	100	87	2	7	3	1	8	2

This comparison is made without consideration of other variables such as type of oil. It is concluded that the concentration of phosphatide alone is not responsible for these reactions and that any variations in stability that resulted from lowering or raising the concentration of phosphatide were not responsible for these reactions.

Concentration of Phosphatide.—Soya-bean phosphatides have been consistently the most efficient emulsifier that we have found among such materials that are suitable for clinical use. All the emulsions used clinically have contained this preparation as the principal emulsifying agent. It has certain disadvantages, among which are its vasomotor effects[14] and its chemical complexity and reactivity.[15] In Table VI is shown an analysis of the effects of altering the concentration of phosphatide upon the incidence of pyrogen response, chills, and colloid reaction. It appears that there is no constant or easily recognizable difference that can be ascribed to the concentration of phosphatide as far as these reactions are concerned.

In Table VII the data are shown indicating the effect of different phosphatide concentrations upon systolic blood pressure during infusion of fat emulsions. There is a definite difference between concentrations of 0.5 per cent and concentrations above 1.0 per cent. Between 1.0 and 2.0 per cent phosphatide concentration there is a further increase in the incidence of vasopressor responses, although not as striking as the difference between 0.5 and 1.0 per cent. It is concluded that the vasopressor response is related in part to the phosphatide and that increasing concentrations of this agent lead to a higher incidence and extent of this undesirable side effect.

TABLE VII. SYSTOLIC BLOOD PRESSURE RESPONSE IN PATIENTS RECEIVING EMULSIONS OF DIFFERENT PHOSPHATIDE CONCENTRATIONS

CONCENTRATION PHOSPHATIDE (%)	NUMBER PATIENTS	NUMBER INFUSIONS	MILLIMETERS OF MERCURY RISE					
			0-10	10-20	20-30	30-40	40-50	50+
			PER CENT OF TOTAL NUMBER OF INFUSIONS					
0.5	59	398	49	22	15	6	4	4
1.0	65	222	22	26	23	19	5	4
1.2	55	256	25	16	23	10	13	13
2.0	21	93	21	18	18	20	10	12

It is concluded that higher concentration of this emulsifying agent is associated with an increased incidence of higher blood pressure rises. This table contains data available as of November, 1953.

TABLE VIII. REACTIONS TO EMULSIONS CONTAINING DIFFERENT SYNTHETIC EMULSIFYING AGENTS IN COMBINATION WITH PHOSPHATIDE

STABILIZER	NUMBER PATIENTS	NUMBER INFUSIONS	DEGREES RISE FAHRENHEIT					CHILLS	COLLOID REACTION
			0-1°	1.1°-2.0°	2.1°-3.0°	3.1°-4.0°	4.1+		
			PER CENT OF TOTAL NUMBER OF INFUSIONS						
Demal-14	105	372	85	7	4	2	2	6	1
Emcol	40	103	85	6	6	1	2	7	5
Pluronic	234	667	84	6	5	3	1	5	2

It is concluded that these emulsifying agents are not in themselves responsible for these reactions.

Synthetic Emulsifying Agents.—In Table VIII the data pertaining to the effect of three different synthetic emulsion components upon pyrogen response are shown. These agents contribute to the ease of emulsification and to stability when used in conjunction with phosphatide, although none of them is satisfactory alone. The results indicate that none of these three synthetic agents appreciably alters the incidence of thermogenic reactions.

Table IX summarizes the data on vasopressor effect of these three emulsifiers. Here there are significant differences indicating that Pluronic in particular results in a higher incidence of vasopressor responses than either Demal-14 or Emcol. Table X presents further data on the effect of Pluronic upon systolic blood pressure, and it is obvious that increasing concentrations of this material are associated with a higher incidence of vasopressor response. For this reason the amount of Pluronic has been reduced to a minimum in the emulsions prepared in recent months. Nevertheless, the continued use of Pluronic is warranted because at minimal effective concentrations the vasomotor responses are greatly reduced, while its influence on particle size is retained. It is not known whether its vasomotor action is a direct one or mediated through its effect on the phosphatides.

TABLE IX. SYSTOLIC BLOOD PRESSURE RESPONSE TO EMULSIONS CONTAINING DIFFERENT SYNTHETIC EMULSIFYING AGENTS IN COMBINATION WITH PHOSPHATIDE

STABILIZER	NUMBER PATIENTS	NUMBER INFUSIONS	MILLIMETERS OF MERCURY RISE					
			0-10	10-20	20-30	30-40	40-50	50+
			PER CENT OF TOTAL NUMBER OF INFUSIONS					
Demal-14	101	477	33	28	19	10	5	5
Emcol	27	83	46	19	12	11	6	6
Pluronic	95	454	22	19	22	13	11	11

These data suggest that in addition to the phosphatide, synthetic emulsifying agents can be responsible for blood pressure rises. Pluronic in particular can be incriminated. Nonspecific effects of these agents may be responsible, e. g., in vivo stability, particle charge, and alterations at the oil-water interface.

TABLE X. SYSTOLIC BLOOD PRESSURE RESPONSE DURING INFUSION WITH EMULSIONS CONTAINING PLURONIC

CONCENTRATION PLURONIC (%)	NUMBER PATIENTS	NUMBER INFUSIONS	MILLIMETERS OF MERCURY RISE					
			0-10	10-20	20-30	30-40	40-50	50+
			PER CENT OF TOTAL NUMBER OF INFUSIONS					
0.2	45	220	33	21	19	12	8	7
0.5	47	171	20	16	28	17	11	7
1.0	19	90	14	11	19	9	19	28

This comparison is made without regard to other variables such as oil and concentration of the phosphatide. There seems to be a clear-cut correlation between the incidence of vasopressor responses and increasing concentration of this agent.

TABLE XI. INFLUENCE OF TOTAL SERUM PROTEIN CONCENTRATION ON REACTIONS TO INTRAVENOUS FAT

TOTAL SERUM PROTEIN (GRAMS PER CENT)	NUMBER INFUSIONS	DEGREES RISE FAHRENHEIT					CHILLS	COLLOID REACTION
		0-1°	1.1°-2.0°	2.1°-3.0°	3.1°-4.0°	4.1°+		
		PER CENT OF TOTAL NUMBER OF INFUSIONS						
Over 6.0	375	82	7	3	4	0	5	2
Under 6.0	414	86	6	4	2	2	6	1

This comparison does not take into consideration that many different batches and types of emulsion were used. There is no significant difference between the two groups of patients.

Serum Protein Levels of Patients.—One of the possible explanations for the fact that 5 to 15 per cent of our infusions have resulted in pyrogenic reactions of varying degree is that individual abnormalities of certain patients are responsible. Inasmuch as the phospholipid used in these emulsions reacts with blood proteins,[15] and as it has been noted that some severe reactions have occurred in patients with low serum protein levels, the data in regard to levels of total

serum protein, albumin, and globulin and to albumin-globulin ratios have been examined. In Table XI is shown the incidence of pyrogen responses to infusion of emulsified fat in patients with total protein levels above 6.0 Gm. per 100 ml. of serum as compared with pyrogen responses in patients with total protein levels below 6.0 Gm. per 100 ml. of serum. There is no apparent difference between these two groups of patients. Similarly, although the data are not presented here, the presence of reversed albumin-globulin ratios was not found to correlate with the occurrence of pyrogen responses. Hence, it is concluded that neither the levels of total protein, albumin, or globulin, nor the albumin-globulin ratio of the patient's serum determines whether or not a thermogenic reaction will occur.

TABLE XII. INFLUENCE OF PREOPERATIVE AND POSTOPERATIVE STATUS OF PATIENTS UPON INCIDENCE OF REACTIONS TO INTRAVENOUS FAT

	NUMBER PATIENTS	DEGREES RISE FAHRENHEIT					CHILLS	COLLOID REACTION
		0-1°	1.1°-2.0°	2.1°-3.0°	3.1°-4.0°	4.1°+		
		PER CENT OF TOTAL NUMBER OF INFUSIONS						
		First Infusions						
Preoperative	77	71	10	10	4	4	6	4
Postoperative	88	64	17	12	2	4	12	2
		Second Infusions						
Preoperative	77	78	10	8	3	1	4	0
Postoperative	88	66	17	11	3	2	9	0
		Third Infusions						
Preoperative	77	73	12	7	5	5	3	0
Postoperative	88	68	16	3	8	4	12	0

Incidence of fever, chills, and colloid reaction in preoperative and postoperative patients who received at least 3 infusions per patient. The postoperative group had a higher incidence of febrile response and chills. It is concluded that the 2 groups of patients respond differently and that the unknown differences may be related to the problem of pyrogenicity.

Qualitative Abnormalities of Serum Proteins.—It is known that surgery, infection, malignancy, and trauma may cause a change in the proteins of the blood.[16-19] In addition to protein changes, there are other substances such as polysaccharides which appear in the blood under similar conditions.[19, 20] Under some circumstances in vitro these alterations adversely affect colloids introduced into such sera.[21] To test this possibility the patients were divided according to their preoperative or postoperative status, and the results of the first three infusions compared. The results of this comparison are shown in Table XII. The differences are not striking except that 2 to 6 times as many postoperative patients had chills and the incidence of thermogenic responses was higher in the postoperative group. In view of the fact that a majority of the preoperative patients had conditions that might be accompanied by similar changes in the blood, these differences may be important. If so, charge phenomena leading to agglutination of fat particles might be significant in regard to pyrogen effects of fat infusion. It seems plausible that phenomena such as platelet or red cell agglutination may also be involved. A similar suggestion has recently been made by Becker and Grossman[22] from consideration of experimental data relative to physical characteristics of fat emulsions.

The Effect of Pyribenzamine.—It has been found that reactions to the infusions of blood can be reduced by the administration of antihistaminics.[23] It

seemed possible that a similar effect might result from the use of such drugs during administration of emulsified fat. Twenty-five milligrams of pyribenzamine was added directly to each 600 ml. bottle of fat emulsion and 185 infusions made on 65 patients. Twenty different batches of emulsions were used in this experiment. In this group of patients no significant change in the incidence of pyrogen reactions resulted.

Febrile Patients.—During the collection of the data described previously, 326 infusions were made on 83 febrile patients. These patients were severely ill, usually from intraperitoneal infection. In this group of patients it was impossible to analyze the data in the same manner as for nonfebrile patients. Therefore, each infusion was classified as "probably a reaction" or "probably no reaction" on the basis of the patient's temperature chart preceding the first infusion. Using this method, 91 per cent of the infusions were classified as "probably no reaction." Several factors obviously weight the data favorably. For example, many of the infusions were carried out daily in patients who tolerated the fat well, this being indicated for the patient's nutrition. In this group of febrile patients there are included several who received fat infusions daily for over one month. Also, reaction to the first infusion in very sick patients was usually considered sufficient reason to abandon further attempts at fat infusion.

COMMENT

The results presented here demonstrate that emulsified fat can be successfully given to a large group of patients with safety and with a minimum of undesirable side effects. The recent experience with purified cottonseed and synthetic oils is particularly encouraging. They have given the lowest incidence of pyrogen reactions of any type of emulsion tested, approximately 3 and 6 per cent, respectively, if temperature rises up to 2.0° F. are excluded (see Table I).

It is quite obvious from these studies that the cause of the pyrogen reactions remains uncertain. The problem is complicated by the fact that in the majority of patients no rise in temperature occurs. This makes it necessary to have a large number of infusions to evaluate experimental modifications in the composition and preparation of emulsions. At the present time approximately 1 infusion out of 20 results in a rise of more than two degrees. From the beginning of this work it has been felt that when febrile responses occurred, the fault lay with the emulsions. However, it is possible that the patients reacting to fat infusions are different in some way and that for general use there is nothing basically wrong with the emulsions as they are currently prepared.

The evidence pertaining to the role of phosphatide and Pluronic in causing vasomotor responses seems conclusive. However, there is another possibility that deserves consideration, namely, that these vasopressor responses are yet another manifestation of the effect of intravenous administration of colloids, and as such may require as much consideration as the more dramatic and more serious reactions described under colloidal phenomena. Both phosphatide and Pluronic are used because they contribute to stability and ease of emulsification. The manner in which emulsion components are aligned at the lipid-water interface may also have a bearing on the problems of pyrogenicity and colloid reactions.

TABLE XIII. COMPARISON OF OLIVE AND COTTONSEED OIL EMULSIONS

OIL	NUMBER PATIENTS	NUMBER INFUSIONS	NUMBER INFUSIONS PER PATIENT	DEGREES RISE FAHRENHEIT					CHILLS	COLLOID REACTION
				0-1°	1.1°-2.0°	2.1°-3.0°	3.1°-4.0°	4.1°+		
				PER CENT OF TOTAL NUMBER OF INFUSIONS						
				All Infusions						
Olive	76	181	2.4	68	17	8	4	1	7	1
Cottonseed	132	252	1.9	91	6	2	1	0	4	2
				First Infusion						
Olive	37	37	1	59	24	11	0	3	11	0
Cottonseed	60	60	1	83	8	7	2	0	10	5
				Second Infusion						
Olive	26	26	1	58	23	11	0	8	11	0
Cottonseed	33	33	1	97	3	0	0	0	3	0

Incidence of pyrogen repsonse, chill, and colloid reactions on infusion of olive oil and cottonseed oil emulsions. Both emulsions were made with phospholipid 1.2 per cent and Pluronic 0.2 per cent as the emulsifying system, and both emulsions contained 10 per cent oil. From a pyrogen standpoint cottonseed is superior and from the standpoint of the colloidal reaction the olive oil preparations were better. See text for further discussion.

From the standpoint of the fineness of visible particle size, the cottonseed oil emulsions are superior to similar olive oil emulsions. In Table XIII it can be seen that comparison of the results of clinical administration shows an inverse relationship between colloid reaction and thermogenic response. The cottonseed emulsions caused fewer pyrogen reactions but an increased incidence of colloid reactions; the olive oil emulsions, while more pyrogenic, caused a lower incidence of colloid reactions. On the basis of these findings, which compare two oils, it is tempting to relate colloidal reactions to pyrogen responses in an inverse manner. However, if one considers the data obtained with cottonseed oil infusions, a higher incidence of pyrogen reactions was associated with an increase in colloid reactions when the data of the first and second infusions are compared.

The general tenor of this report, which stresses the untoward results of intravenous fat infusion, might leave an impression of pessimism, but it should be emphasized that a considerable number of patients and infusions are included and that 80 to 90 per cent of infusions were without adverse reactions. The outlook for solution of the problems associated with the use of emulsified fat is better at present than at any time in the past.

SUMMARY

The results of 1,466 infusions of fat emulsion into 426 patients are presented and discussed. Emulsions containing 10 to 15 per cent of coconut, olive, peanut, and cottonseed oils, and synthetic triolein have been used.

Data pertaining to such reactions as pyrogenicity, blood pressure, pulse, and chills are presented. The collected data are analyzed in regard to the overall effect of type of oil, concentration of oil, concentration of emulsifiers, presence of synthetic emulsifiers, quantitative and qualitative serum protein abnormalities of patients, and the effects of an antihistaminic on these reactions.

Ninety-one per cent of the cottonseed oil infusions, 86 per cent of the coconut oil infusions, 82 per cent of the olive oil infusions, 85 per cent of the coconut oil-olive oil infusions, 83 per cent of the peanut oil infusions, 76 per cent of the synthetic triolein infusions were without pyrogen reaction. If one excludes temperature rises of 2.0° F., the percentage of successful infusions rises appreciably to 97 per cent for emulsions of cottonseed oil and 94 per cent for emulsions of synthetic triolein. A small number of other unfavorable reactions occasionally observed are discussed in detail, especially as they pertain to the intrinsic colloid character of fat emulsions.

Substantial progress has been made toward the solution of the problems associated with the availability and use of emulsions of fat that can be given satisfactorily by vein.

We wish to acknowledge with gratitude the help of numerous enthusiastic research assistants, also to express appreciation for generous quantities of various materials supplied as follows: purified phosphatides, olive oil, and sterile disposable infusion sets from the Upjohn Co., Kalamazoo, Michigan; purified cottonseed oil from the Southern Regional Research Lab., U.S.D.A., New Orleans, Louisiana; Demal-14 and Emcol (oleic and stearic esters of polyglycerol, respectively) from the Emulsol Corp., Chicago, Illinois; Pluronic-F68 (poly-

ethylene-propylene glycol) from the Wyandotte Co., Wyandotte, Michigan; Pyribenzamine from Ciba Pharmaceutical Products, Summit, New Jersey; and synthetic triolein from Emery Industries, Cincinnati, Ohio.

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THE USE OF DIFFERENTIAL C^{14} LABELING FOR THE INVESTIGATION OF THE IN VITRO ANTITUBERCULOUS ACTIVITY OF ISONICOTINYL HYDRAZIDE OF GLUCURONOLACTONE

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ISONICOTINYL hydrazide of glucuronolactone (INH-G) was introduced recently as a therapeutic agent for the treatment of tuberculosis.[1-3] It was claimed that this isoniazid-containing compound (Fig. 1) is at least as effective as isoniazid (INH) and less toxic on a weight-to-weight basis. Furthermore, the isoniazid of glucuronolactone (INH-G) was reported to be active against tubercle bacilli which had become resistant to INH.

The purpose of this study was to evaluate this antituberculous drug by traditional in vitro methods as well as isotope-tracer techniques. In the first portion of the study determinations were made of the concentration of INH-G necessary to inhibit the growth of tubercle bacilli resistant to INH at different levels.

The tracer studies were done with C^{14}-labeled glucuronolactone which was synthesized with unlabeled isoniazid to produce isoniazid of C^{14} glucuronolactone (INH-C^{14}G). Earlier studies in this laboratory[4] indicated that INH-sensitive tubercle bacilli take up and retain significant amounts of C^{14}-labeled INH, whereas resistant bacilli do not. It was reasoned that, if INH-G acted as a whole molecule, sensitive and INH-resistant tubercle bacilli should take up the INH-C^{14}G and should become radioactive. If, however, the glucuronolactone were split off and the antituberculous activity of the compound were dependent only upon the isoniazid it contained, then the bacilli would not become radioactive on exposure to INH-C^{14}G because only the glucuronolactone portion of the molecule would be labeled.

MATERIAL AND METHODS

Throughout the experiments human tubercle bacilli, strain H37Rv, were used. For some experiments these originally INH-sensitive strains were made resistant to INH at different levels by passing them successively through liquid media containing increasing amounts of INH. Strains with the following resistance levels were used: between 1 and 2 μg, 7.5 and 10 μg, and 20 and 30 μg of INH per milliliter liquid medium. The medium used throughout the experiments was Dubos liquid Tween*-albumin medium.[5] Growth of the tubercle bacilli was measured in calibrated test tubes in the Coleman nephelometer and expressed in nephelometric units.[6] Each result represents the average of readings of at least nine tubes.

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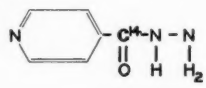
*Proprietary name of the polyoxyethylene derivative of Sorbitan monooleate, obtained from Atlas Powder Co., Wilmington, Del.

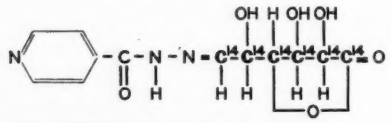
In the experiments in which the uptake of radioactive drugs by the tubercle bacilli was determined, the drugs were added seven days after the inoculation and left in contact with the bacilli for another seven days. The cultures were then heated at 60° C. for 30 minutes and the bacilli separated from the medium by centrifugation. The organisms were washed three times with water containing 0.02 per cent Tween 80 and finally suspended in water with 0.02 per cent Tween 80. One milliliter of this suspension was spread on weighed copper planchets 10 cm.^{2} in area and the radioactivity measured in an internal continuous gas flow counter.[4] The results are expressed in micrograms of the labeled drug $\times 10^{-3}$ per milligram bacilli (desiccated weight[6]).

The unlabeled drugs used in the experiment were isoniazid (INH) and isoniazid of glucuronolactone (INH-G).* The latter compound contains 0.44 μg of INH per microgram of INH-G.

The radioactive drugs used were the following: C^{14}-labeled isoniazid (C^{14}INH),† synthesized by Murray and Langham,[7] with the C^{14} in the carboxyl position (Fig. 1), glucuronolactone uniformly labeled with C^{14} (C^{14}G),‡ and the compound of unlabeled isoniazid with radioactive glucuronolactone (INH-C^{14}G)‡ (Fig. 1).



ISONIAZID



ISONIAZID OF GLUCURONOLACTONE

Fig. 1.—Formulas of isoniazid and isoniazid of glucuronolactone, indicating the labeled carbon atoms.

RESULTS

Tubercle bacilli, strain H37Rv, with a resistance level between 1 and 2 μg of INH per milliliter did not grow in medium containing 2 μg of INH per milliliter, while their growth was identical in INH-free medium and in medium containing 1 μg of INH per milliliter (Fig. 2). This confirmed the resistance level between 1 and 2 μg of INH per milliliter. While 2 μg of INH-G, containing 0.88 μg of INH, did not inhibit growth at all, 4.6 μg of INH-G, containing 2 μg of INH, exerted an inhibition, which was later overcome by the bacilli (Fig. 2).

Bacilli with a resistance level between 7.5 and 10 μg of INH per milliliter were not inhibited by 10 μg of INH-G containing 4.4 μg of INH or by 20 μg of INH-G containing 8.8 μg of INH. No growth occurred in medium with a concentration of 30 μg of INH-G per milliliter, corresponding to 13.2 μg of INH.

Bacilli with a resistance level between 20 and 30 μg of INH per milliliter were not inhibited by 50 μg of INH-G containing 22 μg of INH, but were inhibited by 100 μg of INH-G containing 44 μg of INH.

*We are indebted to Barnes-Hind Laboratories for letting us have this material.

†The C^{14} used for the synthesis of this compound was obtained on allocation from the Isotope Division, United States Atomic Energy Commission.

‡We are indebted to the Corn Products Refining Company, Argo, Ill., for supplying this material.

In the experiments employing labeled drugs against INH-sensitive bacilli (Fig. 3) it was shown that C^{14} INH is taken up by the organisms, whose growth is stopped. C^{14} glucuronolactone does not inhibit growth and is not taken up. The isoniazid of C^{14} glucuronolactone in two different concentrations has inhibitory activity equal to the C^{14} INH, but the labeled part of the compound is not taken up by the bacilli.

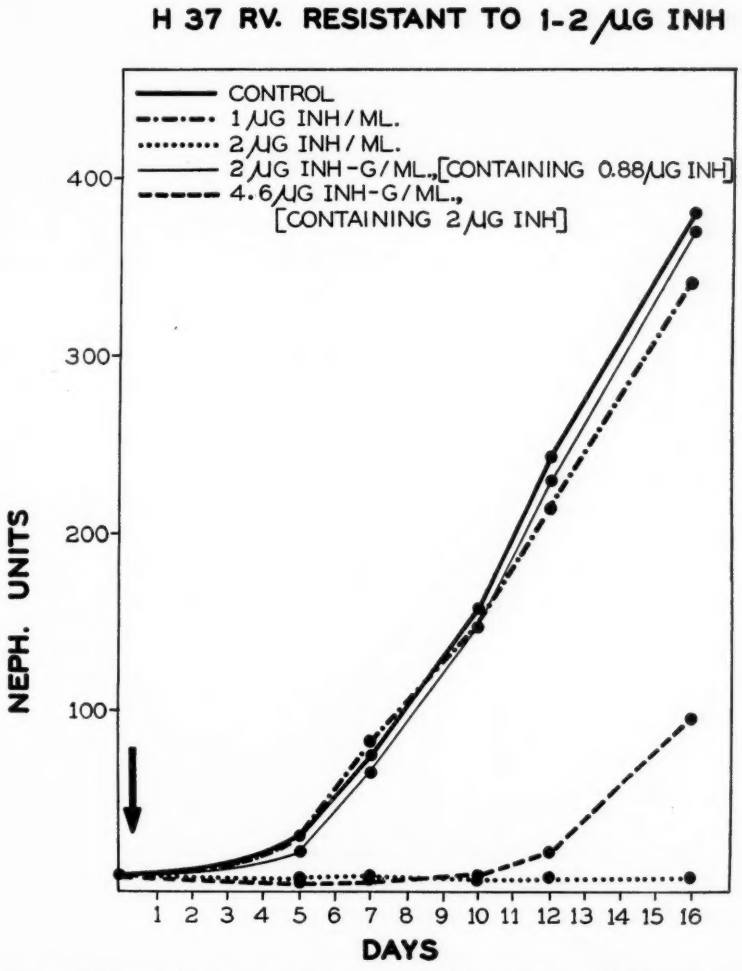

H 37 RV. RESISTANT TO 1-2 µG INH
CONTROL
1 µG INH / ML.
2 µG INH / ML.
2 µG INH-G / ML., [CONTAINING 0.88 µG INH]
4.6 µG INH-G / ML., [CONTAINING 2 µG INH]
NEPH. UNITS
400
300
200
100
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
DAYS

Fig. 2.—Comparison of the growth-inhibitory effect of different concentrations of isoniazid and isoniazid of glucuronolactone upon isoniazid-resistant tubercle bacilli. The drugs were added at the time of inoculation.

As previously described, INH-resistant bacilli (Fig. 4) treated with C^{14} INH grow at the same rate as untreated controls and take up insignificant amounts of the drug. INH-C^{14}G fails to inhibit the growth of these bacilli, and they do not take up any of the radioactive compound.

DISCUSSION

The data presented indicate that isoniazid of glucuronolactone is ineffective against isoniazid-resistant tubercle bacilli. Only when the amount of INH

in the compound surpasses the level of resistance is the drug able to inhibit growth of INH-resistant organisms. This result was obtained at three different levels of resistance.

Further proof that this compound is not effective against INH-resistant bacilli is offered by the experiments with labeled drugs. The fact that INH-sensitive bacilli become radioactive after exposure to carbon-labeled INH, while INH-resistant bacilli do not, suggests that this drug has to be taken up and retained by the organism in order to be effective. Our experiments show that the INH-$C^{14}G$ is not taken up. They indicate also that even sensitive bacilli, whose growth is inhibited by the compound, do not become radioactive. One has to conclude that isoniazid of glucuronolactone does not affect the tubercle bacillus as an intact compound. It is effective only through the isoniazid contained in and probably released from its molecule. It is therefore not active against isoniazid-resistant organisms unless the amount of isoniazid in the molecule surpasses the level of resistance of this strain.

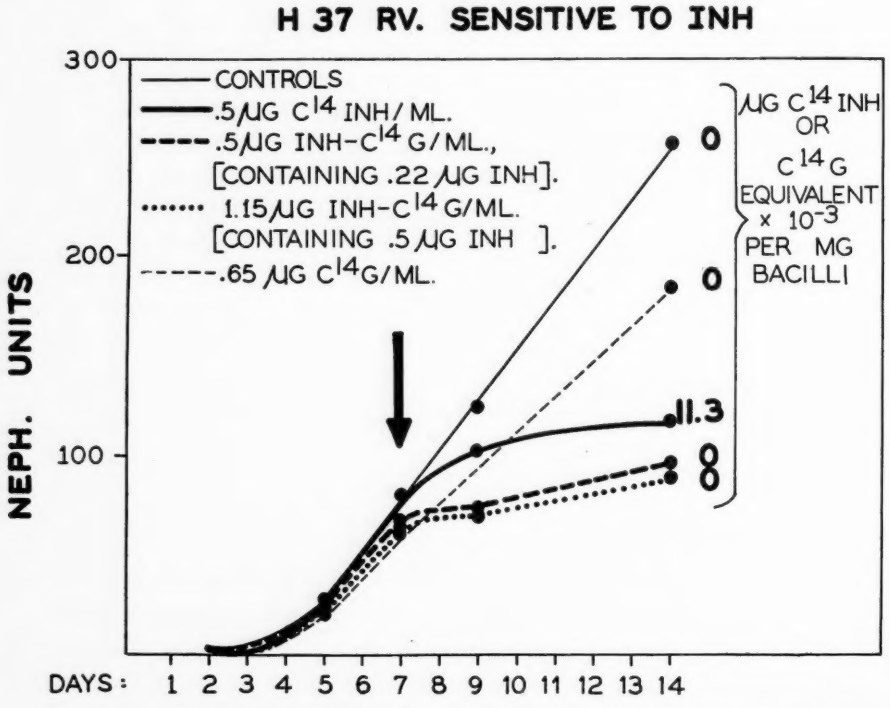

H 37 RV. SENSITIVE TO INH
300
200
100
NEPH. UNITS
CONTROLS
.5 µG C14 INH/ML.
.5 µG INH-C14 G/ML., [CONTAINING .22 µG INH].
1.15 µG INH-C14 G/ML. [CONTAINING .5 µG INH].
.65 µG C14G/ML.
µG C14 INH OR C14 G EQUIVALENT x 10-3 PER MG BACILLI
0
0
11.3
8
0
DAYS: 1 2 3 4 5 6 7 8 9 10 11 12 13 14

Fig. 3.—Growth of and uptake of radioactivity by isoniazid-sensitive tubercle bacilli after the addition of C^{14} isoniazid, C^{14} glucuronolactone, and isoniazid of C^{14} glucuronolactone. The drugs were added 7 days after inoculation, and the radioactivity was measured after 7 more days and is indicated by the numbers at the right side of the graph.

We have previously shown[4] that the growth inhibition of tubercle bacilli by INH in vitro is not proportional to the concentration of the drug in the medium. Once the minimal inhibitory concentration is obtained, a maximal effect is observed. If the growth of a given suspension of tubercle bacilli in

liquid medium is inhibited, for example, by 0.1 μg of INH per milliliter, even the addition of 100 μg of INH per milliliter will not produce any additional inhibitory effect. This explains why, with the relatively large doses of INH-G and INH used by the French authors in their in vitro studies,[2] identical effects on a weight-to-weight basis were obtained.

It seems possible that the glucuronolactone has a detoxifying action upon isoniazid, in the same manner as it has upon other substances[8-10] and, therefore, the compound might be less toxic than INH alone. Since, however, the usual doses of isoniazid have proved to be of neglible toxicity, and since it is doubtful if higher doses are desirable, this seems to be of no practical importance.

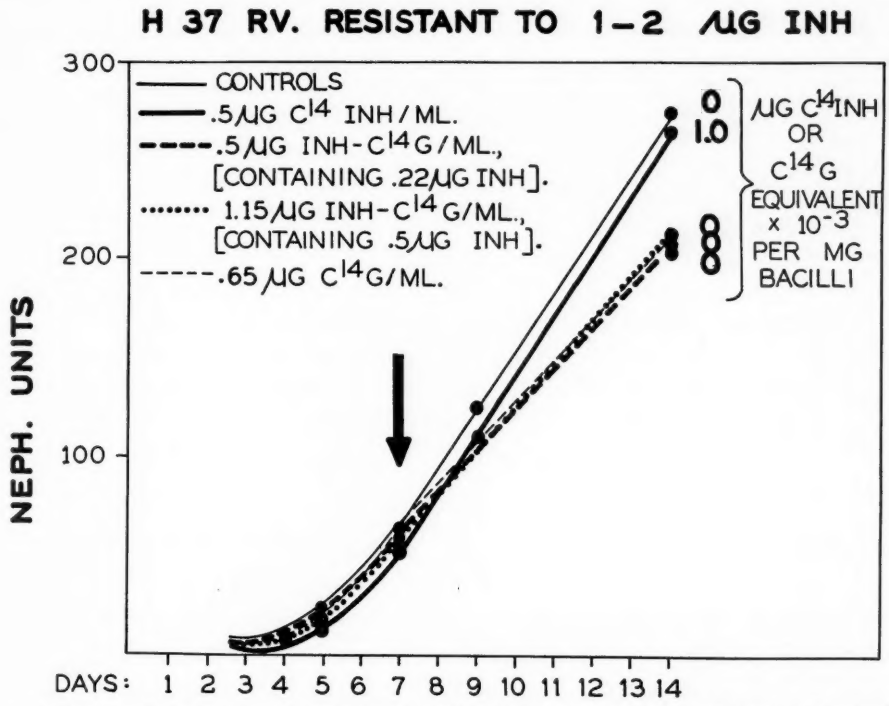

H 37 RV. RESISTANT TO 1–2 μG INH
300
200
100
NEPH. UNITS
CONTROLS
.5 μG C14 INH / ML.
.5 μG INH-C14 G / ML., [CONTAINING .22 μG INH].
1.15 μG INH-C14 G/ML., [CONTAINING .5 μG INH].
.65 μG C14 G/ML.
0
1.0
8
8
μG C14 INH OR C14 G EQUIVALENT x 10-3 PER MG BACILLI
DAYS: 1 2 3 4 5 6 7 8 9 10 11 12 13 14

Fig. 4.—Growth of and uptake of radioactivity by isoniazid-resistant tubercle bacilli after the addition of C14 isoniazid, C14 glucuronolactone, and isoniazid of C14 glucuronolactone. The drugs were added 7 days after inoculation, and the radioactivity was measured after 7 more days and is indicated by the numbers at the right side of the graph.

SUMMARY

An investigation of the antituberculous activity of isonicotinyl hydrazide of glucuronolactone was made by traditional in vitro methods and by isotope-tracer studies. It revealed that this compound is as active in vitro against virulent tubercle bacilli as the amount of isoniazid contained in the molecule. The compound was not effective against isoniazid-resistant bacilli. It was concluded that isoniazid of glucuronolactone does not affect the tubercle bacillus as an intact compound, but probably releases the isoniazid.

The possibility of a detoxifying action of the glucuronolactone upon isoniazid was not excluded.

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THE TAGGING OF LEUKEMIC LEUKOCYTES WITH RADIOACTIVE CHROMIUM AND MEASUREMENT OF THE IN VIVO CELL SURVIVAL

MARY SUE MCCALL, B.S., DONALD A. SUTHERLAND, M.D., ANNA M. EISENTRAUT, AND HENRY LANZ, PH.D.
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THE life span of normal human leukocytes has been estimated at approximately fourteen days.[1, 2] This value was determined by measuring the rise and fall of the nuclear P^{32} tagged desoxyribosenucleic acid fraction. This DNA fraction was considered to be fixed during the life of the cell and was released only with lysis of the leukocyte. Nine of the fourteen days represent the intravascular or extra-marrow phase of the life span.[2] Osgood measured the life span of various leukemic leukocytes with a similar technique and reported that the granulocytes of chronic myelogenous leukemia had a life span of three days. He found the lymphocytes in chronic lymphatic leukemia to have a life span of approximately 30 days, while those of acute lymphatic leukemia were observed to have a life span of less than one week.[3] The life span of erythrocytes has been measured using radioactive chromium and with either an in vitro or in vivo tagging technique.[4-6] The tagging of leukocytes with radioactive chromium has not been described. This report relates our results in tagging leukemic leukocytes with $Na_2Cr^{51}O_4$* and observations on their intravascular life span.

MATERIALS AND METHODS

The clinical material consisted of seven patients with chronic myelogenous leukemia, and four patients with chronic lymphatic leukemia.

A. *The in Vitro Tagging Method.*—The needles, syringes, and glassware were all sterile and siliconized; centrifugation was done at room temperature unless the temperature is specified. Fifty to one hundred milliliters of venous blood was collected in a syringe containing 10 ml. of ACD solution† and in some instances a one-tenth volume of Dextran‡ (used to accelerate the sedimentation of the erythrocytes). The filled syringe was placed in a vertical position until the erythrocytes settled, which usually required two to three hours. A needle was placed on the syringe and bent into a curve so that the supernatant plasma rich in leukocytes could be transferred to a sterile siliconed tube by carefully raising the plunger. The plasma and leukocytes were separated by centrifugation and the cells resuspended in normal saline containing radioactive sodium chromate. The leukocytes were incubated for 20 minutes at 37° C., and the supernatant saline was removed. The cells were then washed until the washing solution removed from the cells was free of radioactivity; this usually required three washings with 30 ml. volumes of a chilled ACD solution. The

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*Obtained from the Radioactive Pharmaceutical Division, Abbott Laboratories, Oak Ridge, Tenn.

†Sterile buffered dextrose acid sodium citrate solution as used in donor transfusion bottles.

‡Expandex, Injection, Commercial Solvents Corporation.

tagged cell suspension was then examined with a phase microscope to determine the ratio of the leukocytes to erythrocytes and the presence of motility of the cells or the intracellular organelles. A tagged leukocyte preparation was considered to be satisfactory if the leukocyte to erythrocyte ratio was at least 50:1 and if amoeboid or intracellular organelle motion was detected. The tagged cells which were injected as part of a life-span study were suspended in either saline or plasma immediately before injection.

B. *The in Vivo Tagging Technique.*—Three hundred to five hundred microcuries of radioactive sodium chromate were diluted in 20 ml. of normal saline and injected intravenously.

C. *Methods Used in Measuring the in Vivo Circulation of Cr^{51} Tagged Leukocytes.*—Venous blood samples were usually obtained at ten minutes, one hour, and twenty-four hours after the tagged cells were injected, and then at daily intervals until the concentration of radioactivity in the samples approached the background of the scintillation well-type crystal unit used. The leukocytes were separated from erythrocytes and plasma, and the Cr^{51} activity associated with the leukocytic fraction was measured in a scintillation well-type crystal unit. The gamma-sensitive Na-I thallium-activated scintillation crystal unit had a background of 2.4 counts per second and 1 microcurie of Cr^{51} in a 4 ml. volume recorded 1,400 counts per second. The intravascular life span of Cr^{51} tagged leukocytes was estimated by relating time to the per cent of persisting cells in a manner similar to that used in the Ashby technique for measuring survival of erythrocytes. The Cr^{51} radioactivity (counts per second) of each sample of isolated leukocytes was expressed as per cent of the maximum activity sample. Corrections were made for the physical decay of the isotope and for any variations in the mass of the isolated leukocytes. The sample showing maximum activity following the injection of tagged leukocytes was at ten minutes, whereas the leukocytes were maximally tagged at one hour after the intravenous injection of $Na_2Cr^{51}O_4$ (in vivo tagging).

Two methods were used to isolate the tagged leukocytes in the study of the intravascular life span.

1. The isolation of the leukocytes was accomplished by layering the blood with high-speed centrifugation in a celluloid test tube; freezing the layered sample in a dry-ice acetone bath; and cutting apart the leukocytic fraction with a power saw. This technique was simple and readily accomplished when the buffy coat formed a wide band as in leukemic patients. Freezing to −60° C. with dry-ice and acetone minimized melting of the blood due to the heat of friction of the saw. A rough approximation of a similar mass of leukocytes could be obtained from different samples of blood from the same patient by standardizing the layering procedure and the size of the frozen segment of leukocytes.

2. A leukocyte-rich plasma fraction was obtained as described in the method for in vitro tagging except that the sedimentation of the erythrocytes and the separation of the leukocytes from the plasma was carried out at 20° C. The leukocytes were washed twice with a 1:16,000 dilution of saponin in ACD solution. The cells were suspended in the saponin solution for 30 to 60 minutes before centrifugation as part of the second washing. Hemolysis was usually complete with the second washing and any free hemoglobin was removed by a final washing with a chilled (20° C.) ACD solution. The tagged leukocytes were packed down in a calibrated centrifuge tube by centrifuging at 2,000 r.p.m. for 15 minutes and the volume of packed cells noted. The cells were lysed with one normal sodium hydroxide and the volume raised to 4 ml.

RESULTS

1. *The in Vitro Tagging of Leukemic Cells.*—The myeloid cells in seven cases of chronic myelogenous leukemia, and the lymphocytes from four cases of chronic and one case of acute lymphatic leukemia have been tagged with $Na_2Cr^{51}O_4$. The per cent uptake of Cr^{51} by the leukocytes varied from 5 to 85 per cent. The differences in the per cent uptake of Cr^{51} by the leukocytes from different patients did not correlate with either the cellular morphology or the

clinical status. The uptake values were obtained with identical intervals of incubation, one hour, and the same temperature of incubation, 37° C. Although there were slight variations in the mass of the leukocytes incubated and also the specific activity of the sodium chromate, there was no apparent consistent effect produced by these two variables.

2. *The Cr^{51} Binding to the Leukemic Granulocytes.*—The type of attachment developed by $Na_2Cr^{51}O_4$ within the immature granulocytes was found to be relatively resistant to the mechanical trauma of repeated washing. However, if the tagged leukocytes were allowed to stand for several hours, the supernatant fluid contained radiochromium. This radiochromium was not bound to protein or cellular debris as shown by dialysis and precipitation of the protein fraction with trichloracetic acid. This "free" Cr^{51} may occur as a result of the action of hydrolytic enzymes activated by cell lysis. The supernatant from tagged leukemic leukocytes broken mechanically, or by freezing and thawing, has been incubated with erythrocytes and leukemic granulocytes, whereupon tagging was not detected. Cr^{51} tagged erythrocytes, hemoglobin, and plasma proteins have been incubated with saline suspensions of leukemic granulocytes, and no Cr^{51} tagging of the leukocytes has been found.

3. *The Effect of Temperature on the Tagging of Immature Granulocytes With $Na_2Cr^{51}O_4$.*—Venous blood was obtained from a patient with chronic myelogenous leukemia and the leukocytic fraction was separated as previously described. The leukocytic suspension was divided into three fractions. The three samples were equilibrated at the various temperatures (37°, 25°, 15° C.) for 30 minutes prior to adding $Na_2Cr^{51}O_4$. The samples were then incubated for one hour; following incubation the cells were washed as previously described. A wet preparation and a stained smear of the washed cells showed them to be morphologically intact. The amount of radioactivity in the three aliquots of tagged cells varied. The following values for per cent of incubated activity bound to the cells were obtained: 37° C. = 17.2 per cent; 25° C. = 8.5 per cent; 15° C. = 3.9 per cent.

4. *The Circulation of Autotransfused Leukemic Granulocytes Tagged in Vitro.*—The circulation of in vitro tagged leukocytes following intravenous injection has been measured on five occasions in a study of three leukemic patients. A consistent finding is that only 16 to 20 per cent of the injected Cr^{51} was found in the intravascular space ten minutes after injection of the cells. Scanning the body at various periods of time after injection of the tagged cells with a scintillation crystal has revealed high concentrations of Cr^{51} in the area of the spleen, liver, and lungs. The relative activity in each organ remained the same over a period of weeks in the leukemic patient studied.

No reactions occurred as a result of the autotransfusions of the washed cells; there were no changes in the leukocyte counts following the five autotransfusions.

One of the autotransfusion studies was done in a patient with chronic myelogenous leukemia having massive splenomegaly, anemia (9.0 Gm. per cent),

leukocytosis (500,000 per cubic millimeter), and marked weight loss. Two 100 ml. blood samples were processed to isolate the leukocytes. Blood for the second isolation procedure was collected immediately before injecting the first tagged cell suspension which contained a white to red blood cell ratio of 15 to 1. The second isolation of leukocytes was more effective and produced a 100 to 1 ratio of white to red blood cells. The second mass of tagged leucocytes was

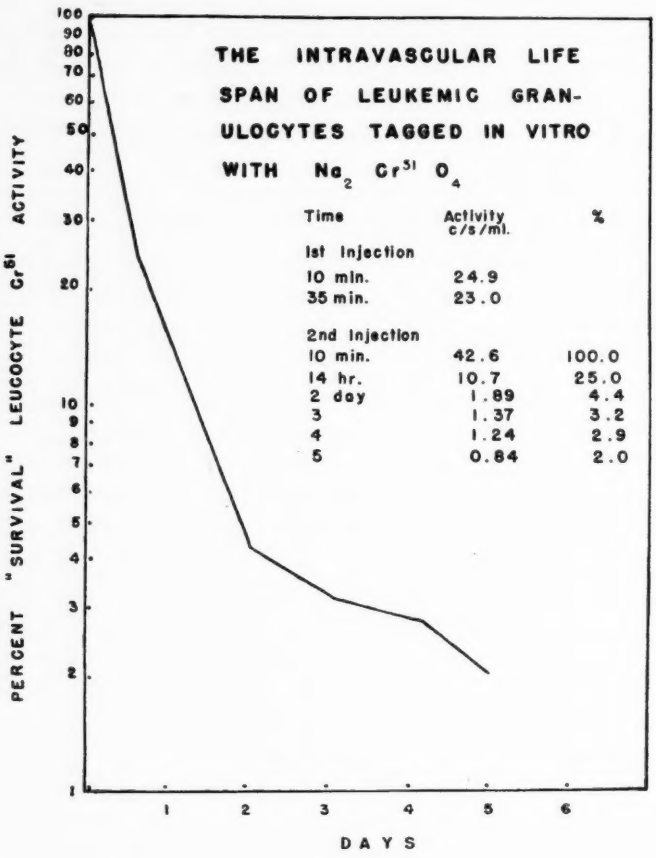

Fig. 1.

injected three hours after the first autotransfusion. The rise in the concentration of tagged leukocytes as a result of the second injection is seen in the activity data listed in Fig. 1. The disappearance of the autotransfused cells was extremely rapid with the initial disappearance rate of $T_{½}$ = 8 hours. The cells remaining after 24 hours and 48 hours disappeared at a progressively slower rate. The disappearance rate during the second postinjection day, $T_{½}$ = 60 hours. It is of interest to note that leukocytes from this patient were tagged at a later date by the in vivo technique and showed a linear disappearance curve with a mean intravascular life span of approximately 12 days.

5. The Tagging of Leukemic Leukocytes in Vivo With $Na_2Cr^{51}O_4$ and Their Intravascular Life Span.—Sufficient Cr^{51} was incorporated into circulating leukocytes following an intravenous injection of sodium radiochromate to permit measurement of the life span in seven patients. The tagged lymphocytes in two individuals demonstrate an intravascular life span of approximately 30 days. The disappearance rate of lymphocytes from two other clinically similar cases of chronic lymphatic leukemia are found within the main group of curves. A median curve drawn through the main group of individual curves would indicate a mean life span of leukemic leukocytes of approximately 13 days.

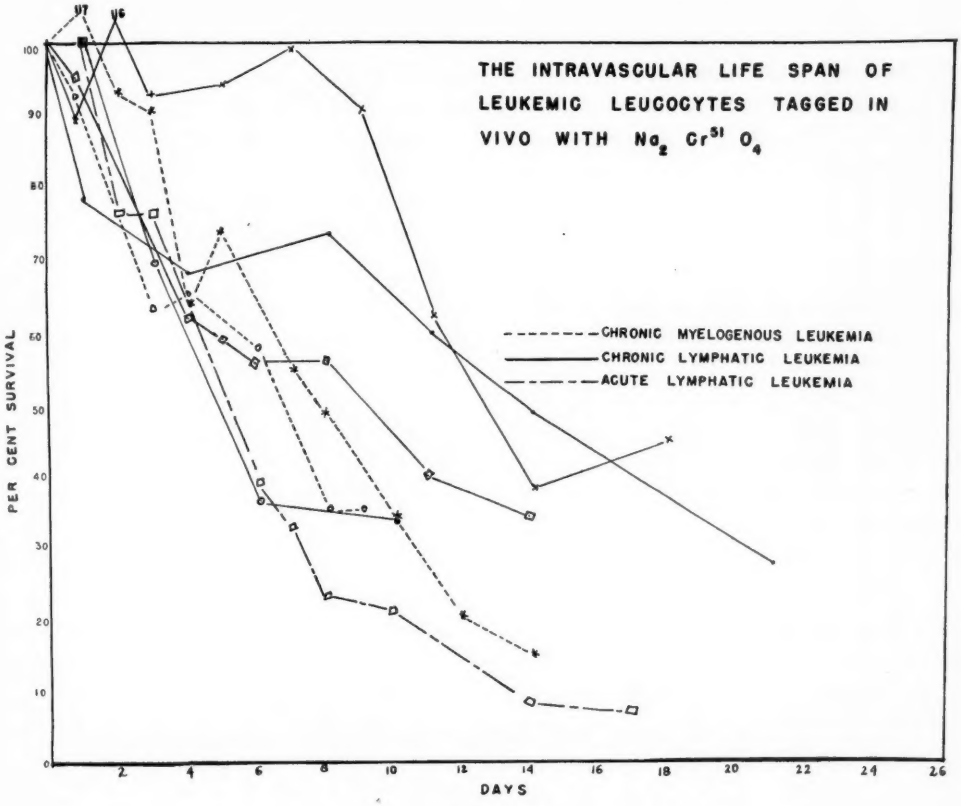

Fig. 2.

DISCUSSION

The life span of leukocytes has been studied in various animals and in normal and leukemic humans. There is a marked variation in the reported values. The wide range of reported values may be due to variations in the following: (1) the method of procuring the leukocytes, (2) the method of measuring life span and cell viability, (3) the species studied (cat, hen, rabbit, rat, and human), and (4) the type of leukocyte studied, normal or leukemic, granulocyte or lymphocyte. The life span values and ancillary data recorded in the literature are listed in Table I. It seems apparent from a review of the

TABLE I. PUBLISHED VALUES FOR THE LIFE SPAN OF LEUKOCYTES

CELL TYPE	AUTHORS	METHOD	SPECIES	LIFE SPAN
I. Lymphocytes	(a) Yoffey[8]	Thoracic duct flow	Cat	10-12 hours
	(b) Adams et al.[9]	Thoracic duct flow	Cat	12-16 hours
	(c) Van Dyke and Huff[10]	X-ray and parabiosis	Cat	3 hours
II. Granulocytes	(a) Ponder et al.[11]	Marrow injury, morphology	Rabbit	2-3 weeks
	(b) Weiskotten[12]	Marrow injury, morphology	Rabbit	3-4 days
	(c) Van Dyke and Huff[10]	X-ray, parabiosis	Rat	23 minutes
	(d) Osgood[13]	Marrow culture	—	61 hours
III. Leukocytes (all forms, nonleukemic)	(a) Otteson and Hevesy[1]	P^{32}—DNA	Human	Gran. 12 days Lymph. 8-30 days
	(b) Lawrence et al.[14]	Feline agranulocytosis virus and cross transfusion	Cat	7.4 hours
	(c) L. P. White[15]	Atabrine tagged cells	Human	30-90 minutes
	(d) Kline and Cliffton[2]	P^{32}—DNA	Human	13.2 days
	(e) Weisberger et al.[16, 17]	Autotransfusion of P^{32} tagged cells:		
		(1) from exudate	Rabbit	No recirculation
		(2) from thoracic duct	Rabbit	No recirculation
	(f) Weisberger and Levine[7]	S^{35}—L-cystine	Human	13 days
IV. Leukemic Cells	(a) Osgood et al.[3]			
	(1) Chronic myelogenous	P^{32}—DNA	Human	3 days
	(2) Acute lymphogenous	P^{32}—DNA	Human	Less than 7 days
	(3) Chronic lymphogenous	P^{32}—DNA	Human	Approx. 30 days
	(b) Bierman et al.[18, 19]			
	(1) Chronic myelogenous	Morphology and cross transfusion	Human	30 hours
	(2) Chronic myelogenous	Morphology and cross transfusion	Human	4 days
	(c) Weisberger and Levine[7]			
	(1) Chronic myelogenous	S^{35}—L-cystine	Human	Slightly less than 13 days
	(2) Chronic lymphatic	S^{35}—L-cystine	Human	Much longer than 13 days
	(3) Acute lymphatic	S^{35}—L-cystine	Human	Much shorter than 13 days

published reports that the life span of human leukocytes has been most adequately estimated by the radioactive phosphorus tagged DNA[2, 3] and radioactive sulfur tagged amino acid studies and that both techniques yielded similar results.[7]

The present study indicates an intravascular life span of approximately 13 days for the leukocytes in chronic myelogenous leukemia, and 10 days in one case of acute leukemia. Two cases of chronic lymphatic leukemia show decay curves similar to those of the patient with myelogenous leukemia. However, the cells from two other patients with chronic lymphatic leukemia had prolonged survival with mean life spans of approximately 30 days. Similar estimates of life span for the respective cell types can be found in the reports of Osgood and associates[3] or Weisberger and Levine.[7] In vitro analyses indicated that there was no reutilization of Cr^{51} by the leukocytes if the Cr^{51} had been previously bound to other leukocytes, erythrocytes, or plasma proteins. The estimates of life span of the various cell types approximate the values reported by authors using different techniques. This suggests that reutilization of the Cr^{51} was not a factor in the in vivo measurements.

The intravascular circulation of leukocytes that have been subjected to removal from their normal habitus has been studied previously in both man and animals.[8-14] Leukocytes obtained from either sterile peritoneal exudate or by catheterization of the thoracic duct of the rabbit did not circulate after return to the vascular compartment but were sequestered by the lung.[16, 17] Similarly, the human lung has been demonstrated to actively trap leukemic leukocytes injected into the intravascular space of cancer patients. The circulation of transfused leukemic cells into leukemic recipients with relative failure of removal of the cells by the lung led Bierman and his associates to suggest that there is a failure of the normal leukocyte trapping mechanism of the lung as a feature of leukemia.[18, 19]

The present study demonstrates the circulation of autotransfused leukemic leukocytes. The majority (80 to 85 per cent) of the Cr^{51} tagged cells injected intravenously was quickly removed from the circulation, but small numbers of the in vitro tagged cells remained in the circulation for several days. This observation was made twice in each of two patients with myelogenous leukemia and on a single study of a third patient with the same disease. Efforts to date to minimize trauma to the cells while removed from the vascular space have not succeeded in increasing the percentage of leukocytes that circulate after return to the vascular compartment.

SUMMARY

1. An in vitro method for tagging leukemic human granulocytes, lymphocytes, and their precursors with radioactive sodium chromate ($Na_2Cr^{51}O_4$) was described. The exact mechanism of the tagging process remains unknown, but was influenced by varying the temperature of incubation.

2. In four instances, such in vitro tagged leukocytes were shown to circulate in vivo following autotransfusion for at least 24 hours. Although

the majority of the cells were rapidly removed from the circulation, in two patients the Cr^{51} activity in the leukocytic fraction was followed and small numbers of tagged leukocytes persisted in the circulation for five days.

3. Leukemic leukocytes have been tagged in vivo by an intravenous injection of radioactive sodium chromate. The leukocytes in chronic myelogenous leukemia had a measured intravascular life span of approximately 13 days. Two cases of chronic lymphatic leukemia demonstrated a similar finding, but the lymphocytes in two additional patients had a life span of approximately 30 days. The cells in one case of acute lymphatic leukemia showed a life span of 10 days.

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COMPLEMENT FIXATION TEST DUE TO THE INTERACTION OF SPECIFIC ANTIPLATELET SERUM AND HETEROLOGOUS PLATELET ANTIGEN

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RECENT observations on platelet immunology have confirmed and suggested the following points[1]: (1) the existence of heteroantigenicity of blood platelets; (2) the iso- and autoantigenicity of blood platelets, as demonstrated in cases of idiopathic thrombocytopenic purpura and in the course of platelet transfusions; (3) the existence in man of platelet groups and types.

The techniques used in these experiments were based on the agglutination of platelets by means of plasmas and/or sera. Several technical improvements have been suggested in order to make the reaction specific and the procedure as simple and reliable as possible. This was accomplished by: (a) employing reagents which avoid a specific platelet agglutination (triton, sequestrene, all-silicone technique, etc.)[2, 3]; (b) analyzing the experimental conditions in which agglutination takes place (pepsinization, addition of calcium and magnesium, of serum)[4]; (c) transforming the platelet agglutination test into a hemagglutination test (use of antigen-coated tanned erythrocytes).[5]

On the basis of theoretical implications, it can be predicted that the interaction of a platelet antibody and a platelet antigen should cause a complement fixation. Evidence of the existence of complement fixation in such conditions is given in this article, with respect to antiplatelet rabbit sera and guinea pig platelets. The possible correlation with human pathology and technical developments is discussed. The influence of heterophile Forssman antibodies was ruled out by adsorption tests.

MATERIAL AND METHODS

1. *Preparation of Antiguinea Pig Platelet Serum.*—Platelet suspensions were obtained from blood of guinea pigs, according to the technique described by Stefanini et al.,[2] Harrington et al.,[3] and ourselves.[6] Platelet concentration was brought up to 4,000,000 per cubic millimeter. Two milliliters of platelet suspension were injected intravenously into rabbits, once a week for six consecutive weeks. Silicone-coated needles and glassware were used for injection. One week after the last injection, the rabbits were bled, the serum was separated from the clotted blood and kept at –20° C. until needed.

2. *Control of Agglutinating Activity of Antiplatelet Serum.*—Agglutination tests were performed by mixing in equal amounts (0.1 ml.) progressive twofold dilutions of serum in saline ranging from 1/2 to 1/512, with guinea pig platelet suspension (2,000,000 per

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cubic millimeter). Serum should be inactivated for 30 minutes at 56° C. and treated with tricalcium phosphate (50 mg. per milliliter). The test can be performed by means of a microtest slide and a cover glass, or by using test tubes. In both cases, an all-silicone technique should be used. Saline and serum of nontreated animals, respectively, are used as controls.

3. *Complement Fixation With Antiplatelet Serum.*—The following reagents were used: (1) rabbit serum before the treatment with guinea pig platelets; (2) antiplatelet rabbit serum; (3) 2.5 per cent suspension of sheep red cells; (4) antisheep hemolysin; (5) fresh guinea pig serum, diluted 1/10; (6) guinea pig platelet antigen.

The rabbit sera were treated as indicated above and then adsorbed with sheep red cells (0.2 ml. per milliliter of serum for 30 minutes at room temperature) and with guinea pig kidney extract (in equal parts for 30 minutes at 37° C.). After each adsorption, the sera were centrifuged. Such a procedure was employed to avoid the interference of Forssman antibodies and of spontaneous anticomplementary activity of rabbit serum.

Sheep red cells were stored at the concentration of 10 per cent in the Alsever solution for a period no longer than 5 days.

Hemolysin and guinea pig serum activities were titrated before being used in the reaction, according to techniques described elsewhere.[7]

Guinea pig platelet antigen was prepared by repeated freezing and thawing of concentrated platelet suspensions (1,000,000 per cubic millimeter). The titration of the platelet antigen was performed according to the scheme of Table I. Two-tenths milliliter of progressive twofold dilutions of platelet antigen (from 500,000 to 62,500 platelets per cubic millimeter) were mixed with 0.2 ml. of guinea pig serum, containing 10 complement units per milliliter and with 0.2 ml. of progressive dilutions of rabbit serum (1/50 up to 1/800) and kept at 37° C. for 60 minutes. Two-tenths milliliter of hemolysin (10 units per milliliter in the final mixture) and 0.2 ml. of a 2.5 per cent sheep red cell suspension were then added to the mixture and left for 30 minutes at 37° C. The antigen unit was considered the amount of antigen contained in 0.2 ml., which is able to inhibit hemolysis in the presence of the highest serum dilution.

The complement fixation reaction was performed according to the same technique, by using 10 units of platelet antigen per milliliter. In our researches, this amount of platelet antigen was contained in a platelet suspension of 125,000 platelets per cubic millimeter. The following controls were prepared with saline: (1) saline for antigen, controlling spontaneous anticomplementary activity of serum in all dilutions; (2) saline for serum, controlling anticomplementary activity of antigen; (3) saline for serum and complement, controlling complementary activity of antigen; (4) saline for serum and hemolysin, controlling hemolytic activity of antigen. Controls should show complete hemolysis. Complement activity should be controlled again at the same time of the final reaction, to avoid an excess or a defect of complement.

Serum activity in the complement fixation reaction was expressed as the reciprocal value of the highest dilution corresponding to total inhibition of hemolysis. It is assumed that the use of a 50 per cent hemolytic complement titration technique would give positivity at higher dilutions than the complete hemolysis end point, as employed in the present experiments and in most complement fixation reactions.

RESULTS AND DISCUSSION

Agglutination tests of guinea pig platelets with antiplatelet rabbit serum have shown a positivity in serum dilution as high as 1/128. The serum of the same animals before the treatment gave negative results. By employing the same sera for the complement fixation reaction, complete inhibition of hemolysis took place at the serum dilution of 1/700. No inhibition of hemolysis was obtained with serum of the same animals before the treatment. This result was

obtained by using sera which had been either adsorbed or not adsorbed with sheep red cells and guinea pig kidney extract, thus ruling out the influence of Forssman antibodies. The other controls (antigen, serum, hemolysin, complement) confirmed the results obtained in the reaction.

The system for titration and control of the platelet antigen is shown in Table I. The results in a typical experiment are illustrated in Table II.

The complement fixation in the presence of specific antiplatelet serum and heterologous platelet antigen supports the concept that complement might be involved in such reactions. In the agglutination tests for detecting drug hypersensitivity (Sedormid, etc.) the presence of complement has been postulated on the basis of experimental data.[8] In other conditions, in which platelet agglutination may be demonstrated in vitro (I.T.P.; experimental production in animals of antibodies against platelets), the presence of complement was not considered to be necessary.

TABLE I. SYSTEM FOR TITRATION AND CONTROL OF PLATELET ANTIGEN

SERUM TITRATION								
TUBE NO.	TEST SERUM (ML.)	DILUTION	COMPLEMENT (10 UNITS) (ML.)	ANTIGEN (10 UNITS) (ML.)	SALINE (ML.)	HEMOLYSIN* (10 UNITS) (ML.)	2.5 PER CENT SHEEP RED CELLS* (ML.)	
1	0.2	1/50	0.2	0.2	0.0	0.2	0.2	Incubate for 30 minutes at 37° C. Read.
2	0.2	1/100	0.2	0.2	0.0	0.2	0.2	
3	0.2	1/200	0.2	0.2	0.0	0.2	0.2	
4	0.2	1/300	0.2	0.2	0.0	0.2	0.2	
5	0.2	1/400	0.2	0.2	0.0	0.2	0.2	
6	0.2	1/500	0.2	0.2	0.0	0.2	0.2	
7	0.2	1/600	0.2	0.2	0.0	0.2	0.2	
8	0.2	1/700	0.2	0.2	0.0	0.2	0.2	
9	0.2	1/800	0.2	0.2	0.0	0.2	0.2	
Serum Controls								
10	0.2	1/50	0.2	0.0	0.2	0.2	0.2	
11	0.2	1/100	0.2	0.0	0.2	0.2	0.2	
12	0.2	1/200	0.2	0.0	0.2	0.2	0.2	
13	0.2	1/300	0.2	0.0	0.2	0.2	0.2	
14	0.2	1/400	0.2	0.0	0.2	0.2	0.2	
15	0.2	1/500	0.2	0.0	0.2	0.2	0.2	
16	0.2	1/600	0.2	0.0	0.2	0.2	0.2	
17	0.2	1/700	0.2	0.0	0.2	0.2	0.2	
18	0.2	1/800	0.2	0.0	0.2	0.2	0.2	
Other Controls								
19	0.0		0.2	0.2	0.2	0.2	0.2	
20	0.0		0.2	0.2	0.4	0.0	0.2	
21	0.0		0.0	0.2	0.4	0.2	0.2	

Incubate for 60 minutes at 37° C.; then add:

*These two reagents should be preincubated for 30 minutes at 37° C.

The data presented in this paper suggest that platelet antibodies can be detected also by means of a complement fixation test. Such findings refer to antiplatelet rabbit sera and guinea pig platelets. The same technique might be applied, under particular circumstances, to human pathology. If so, the

performance of complement fixation, as described above, represents a technical improvement of the previously suggested techniques for identifying platelet antibodies. Owing to the possibility of preparing a standard platelet antigen and of storing it for a prolonged period, it is not necessary to apply all technical procedures for isolating and concentrating platelets in each series of tests. Furthermore, readings of the results proved to be easier by means of complement fixation than for the usual agglutination tests, even when the technique of the antigen-coated tanned erythrocytes is used. In our researches, positivity occurred at a higher serum dilution than in the agglutination tests. This might represent an advantage for diagnostic purposes in human pathology.

TABLE II. RESULTS OF COMPLEMENT FIXATION REACTION IN A TYPICAL EXPERIMENT

Tube No.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
Exp. 1	+	+	+	+	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−	+	+
Exp. 2	+	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+

+ Absence of hemolysis; − complete hemolysis.

In preliminary tests, performed in normal subjects and in cases of essential thrombocytopenia, with and without platelet agglutinating antibodies, it was not possible to detect any positivity of the reaction, i.e., no complement fixation took place in the presence of serum and homologous platelet antigen from normal subjects. Such results are possibly explained by the complexity of the immunologic mechanisms in human pathology, particularly when auto- and isoantigenicity phenomena occur. Work is in progress for explaining the negativity of the complement fixation reaction in these conditions.

SUMMARY

A complement fixation reaction is described for the detection of platelet antibodies by using antiplatelet rabbit serum and guinea pig platelet antigen.

Complete inhibition of hemolysis took place at the serum dilution of 1/700. No inhibition of hemolysis was obtained with serum of the same animals before the immunization.

This result was obtained by using sera which had been either adsorbed or not adsorbed with sheep red cells and guinea pig kidney extract.

Agglutination tests of guinea pig platelets with antiplatelet rabbit serum have shown a positivity in serum dilution as high as 1/128.

Possible correlations with human pathology and technical developments are discussed.

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HEMORRHAGIC DIATHESIS ASSOCIATED WITH HYPERHEPARINEMIA

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IN ADDITION to the thromboplastic deficiencies that give rise to the clinical picture of hemophilia, inhibitors and anticoagulants must also be considered. Differentiation of a hemorrhagic condition clinically indistinguishable from hemophilia, but due to an anticoagulant, is important because of the possibility of successful therapy. In the case of heparinoid anticoagulants, neutralization in vitro and in vivo can be accomplished with such agents as toluidine blue, protamine sulfate, and Polybrene.* The present case is reported because it illustrates the interesting problem of differentiation from hemophilia, the establishment of heparin as the anticoagulant, and the successful use of a heparin neutralizer in therapy.

In 1941, Jaques and Waters[1] demonstrated that the anticoagulant substance responsible for the prolongation of the blood clotting time of dogs in anaphylactic shock was heparin. In addition, these authors were able to show that the heparinoid material isolated from dogs was quite similar to, although not identical with, beef heparin. In 1947, Allen and associates[2] called attention to the peculiarities of the bleeding tendency in thrombocytopenic purpura and postulated that in this condition an increased amount of heparin-like substance was present in the blood. The following year Allen and coworkers[3] described the "heparinemia(?)" which resulted from the x-irradiation of dogs. Further, they claimed that, as in thrombocytopenic purpura, that toluidine blue and protamine sulfate neutralized the heparin-like anticoagulant and resulted in improved clinical control of the hemorrhagic condition. Subsequently these investigators[4-6] described a protamine titration technique to facilitate the study of such patients and added additional laboratory and clinical information relative to the occurrence and therapy of hyperheparinemia. At about the same time Best and Limarzi[7] and Barnard[8] reported a significant increase in heparinoid anticoagulant in a variety of pathologic conditions.

Despite this accumulating evidence relative to the importance of heparinoid anticoagulants in hemorrhagic dyscrasias, many investigators in this field felt that the conclusions were unwarranted, and that true hyperheparinemia occurred very rarely, if at all. Much of this doubt arose from the fact that existing laboratory methods for assay of blood heparin were not fully satisfactory, and that other coagulation defects might easily simulate hyperheparinemia. It was not until 1951 that Bell[9, 10] first described a case

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*Polybrene, Abbott Laboratories, $C_{13}H_{30}Br_2N_2$.

in which there was "a coagulation defect due to an anticoagulant possessing antithromboplastic and antithrombic properties, probably heparin." His careful investigation left little doubt in this particular case that an excess heparinoid anticoagulant was actually present and was responsible for the clinical condition observed. Additional evidence was presented by Crisalli and Cotellessa[11] in a case of congenital hyperheparinemia.

CASE HISTORY

A. S., a 9-year-old boy, was first seen in the Hematology Clinic of the Wadley Research Institute on Sept. 8, 1953. His presenting complaint was that of a bleeding tendency which had been diagnosed previously as hemophilia.

The past history was indeed quite suggestive of hemophilia. On the eighth day after circumcision the patient bled profusely and two blood transfusions were required to check the hemorrhage. No additional episodes were observed until the patient was 14 months old, when hemarthrosis in the left knee occurred without apparent cause. At the age of three, following trauma, he suffered a very extensive subcutaneous hemorrhage which covered the right abdominal wall and the right lower thorax. Actual hemarthrosis apparently occurred only in the first few years of life, but periarticular hematomas without bleeding into the joint had recurred since the age of 5. In early childhood the loss of baby teeth was accompanied by hemorrhages lasting from five to seven days; however, a short time prior to admission to the clinic he had lost a tooth with no evidence of unusual bleeding. In 1951, as a result of injury to the back, he suffered a severe hemorrhage which necessitated seventeen transfusions of fresh whole blood, fresh plasma, and washed red cells. Toluidine blue was also administered during this bleeding episode.

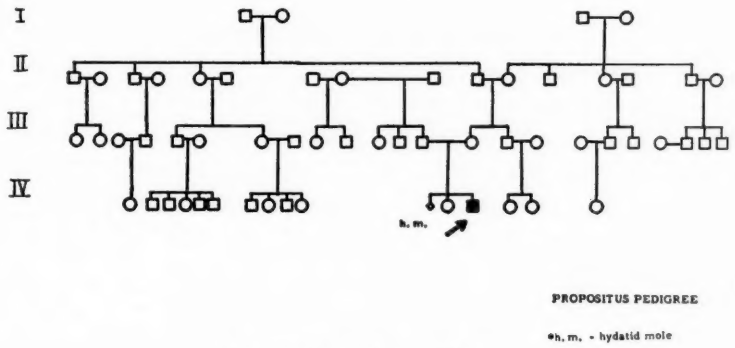

Fig. 1.—Propositus pedigree.

The family history of the propositus is given in Fig. 1. This pedigree is entirely negative in that no member of the family, either maternal or paternal, was known to have a bruising or bleeding tendency. No evidence was elicted that this condition was inherited as a sex-linked recessive characteristic, although such a possibility cannot be rigorously excluded.

Little additional information was derived during the examination of the patient. Ecchymotic areas were noted in both legs; the left knee had a residual hematoma from a recent hemorrhage and was somewhat restricted in motility. Some atrophy of the muscles above the left knee was apparent. Minute spider telangiectases were observed on the right arm and leg. No abnormalities of the chest, heart, or abdomen were noted. Liver and spleen were not palpable; no lymph nodes were felt. The tonsils were enlarged.

Laboratory findings on the first admission in September, 1953, strongly suggested an anticoagulant rather than antihemophilic globulin deficiency as the cause of hemorrhage.

Following these studies, a diagnosis of circulating anticoagulant, possibly heparin, was tentatively made, and toluidine blue therapy was instituted. This diagnosis was confirmed by more complete laboratory studies at a subsequent visit in October, 1954. The complete laboratory findings are discussed in detail under experimental studies.

The results of therapy are of interest in this case. Previous medication had been varied, usually consisting of fresh whole blood at the time of hemorrhagic crisis. On one occasion the patient had received toluidine blue in conjunction with blood and plasma transfusions. Intravenous plasma protein concentrates had also been employed. These concentrates were prepared by freezing freshly collected plasma, thawing until approximately 20 per cent of the plasma sample was fluid, then separating this material from the still frozen remainder. At a very early age the patient was treated with such agents as thromboplastin (subcutaneously and intramuscularly), snake venom, and a "syrup containing horse serum." None of these appeared to be of value. He also had received vitamin B concentrates, vitamin C, and rutin. Following our diagnosis of a circulating anticoagulant, oral toluidine blue* was recommended. After approximately one year, when the patient was seen again, definite clinical improvement was reported. Oral toluidine blue was given only during hemorrhagic episodes. The dose varied from 100 to 150 mg. per day, since vomiting and nausea resulted from doses of 200 mg. Medication at this level was ordinarily continued over a period of four to five days, although the hemorrhages usually subsided within the first twenty-four hours, in contrast to the three- to four-day period of hemorrhage prior to toluidine blue therapy. During this period the patient had shown definite clinical improvement, with reduced frequency and severity of hemarthrosis and other hemorrhagic manifestations. Bruising episodes had occurred at intervals of two to three months, and some bleeding into the tissue around the left knee had been noted on several occasions. No protamine sulfate has been administered to date.†

EXPERIMENTAL STUDIES

Extensive laboratory studies were conducted in an effort to elucidate the nature of the coagulation defect in this case. A general summary of the important findings, together with the normal values as determined in this laboratory, is presented in Table I.

The methods employed in this testing program were as follows:

(1) Whole blood clotting time, Lee and White.[12]

(2) Bleeding time, Duke.[13]

(3) Platelet count, indirect in Reese-Ecker diluent.

(4) "Prothrombin time," Quick.[14]

(5) Prothrombin, Owren.[15]

(6) Proconvertin, Owren.[15]

(7) Proaccelerin, Lewis and Ware.[16]

(8) Fibrinogen and fibrinolysin. One milliliter of oxalate plasma was added to each of two 30 ml. portions of 0.9 per cent saline solution buffered to pH 7.2 with imidazole, mixed, then 1.0 ml. of 2.5 per cent $CaCl_2$ solution and 0.1 ml. of diluted bovine thrombin (10 units total) were added in succession. At the end of one hour the fibrin content of one sample was determined by the biuret method. The second sample was incubated at

*Blutene chloride, Abbott Laboratories, Chicago.

†Subsequent to the writing of this manuscript, a private communication has been received from Dr. Gonda, pediatrition from Mexico City. In mid-November, 1954, A. S. had a severe virus infection with high fever and vomiting. At this time he suffered a serious bleeding episode involving the right hip, thigh, and knee. Oral toluidine blue could not be tolerated because of the vomiting. The patient was given one ampule of protamine sulfate I.V., and although no blood or other anticoagulant therapy was administered, the bleeding stopped promptly, and there was no recurrence of the hemorrhage.

37° C. for 24 hours, and the residual fibrin determined in the same manner. Under these conditions the fibrinolysin (plasma fibrin minus residual fibrin) of normal plasma will lyse less than 20 mg. per cent of the available fibrin.

(9) Capillary fragility test, Dalldorf.[17]

(10) Vitamin C, Roe.[18]

(11) Thrombin clotting time, Biggs and Macfarlane.[19]

(12) Antithrombin, Owen and Bollman.[20] (Results are expressed as thrombin units destroyed per milliliter of plasma, see Table V.)

(13) Protamine titration, Allen and associates.[4]

(14) Heparin determination, Freeman and co-workers.[21] (In this laboratory normal plasmas have ranged in heparin concentration from 4.5 to 18 units per 100 milliliters, with a mean of 10 units per cent.)

(15) Nonspecific test for anticoagulants. This test was conducted as a simple mixing experiment: The patient's plasma and freshly collected normal plasma were mixed in varying proportions and the recalcification time determined. The results are presented in Table II.

TABLE I. SUMMARY OF BLOOD COAGULATION STUDIES

TEST	PATIENT	NORMAL
Lee and White clot time	66′ 15″	4′-15′
Bleeding time	2′ 15″	1′-5′
Quick ''prothrombin time''	22.4″	15.6″
Prothrombin	95 %	100 %
Proconvertin	100 %	100 %
Proaccelerin	91 %	100 %
Fibrinogen	0.352 Gm. %	0.2-0.4 Gm. %
Fibrinolysin	None	None
Platelet count	6.79×10^5	$3\text{-}8 \times 10^5$
Dalldorf test	>15/300 mm. Hg	0-2/300 mm. Hg
Vitamin C	1.2 mg. %	0.5-1.5 mg. %
Thrombin clot time	14.4″	14.4″
Antithrombin	140 units/ml.	56-110 units/ml.
Protamine titration	>0.38 mg.	0.12-0.14 mg.
Heparin determination	30 unit %	9.6 unit %
Nonspecific anticoagulants	Positive	None
Prothrombin utilization	<5 %	70-100 %
Thromboplastin generation	Abnormal	Normal
Platelet thromboplastic factor(s)	>95 %	70-100%
Platelet morphology	Normal	Normal
Capillary microscopy	Inconclusive	Normal

TABLE II. DETECTION OF NONSPECIFIC ANTICOAGULANT

COMPOSITION OF CLOTTING MIXTURES		
NORMAL PLASMA	PATIENT'S PLASMA	RECALCIFICATION TIME
0.5 ml.	None	5.5′
0.4 ml.	0.1 ml	7.0′
0.3 ml.	0.2 ml.	9.0′
0.2 ml.	0.3 ml.	11.5′
None	0.5 ml.	>30′

(16) Prothrombin utilization. The prothrombin consumption during a one-hour incubation of the patient's blood at 37° C. was determined as per White, Aggeler and Glendening[22]; however, greater specificity was achieved by the modifications[23] outlined in Table III. It should be noted in this connection that the AHG concentrate, and the aged serum employed as corrective agents in the modified test are known to contain plasma thromboplastin antecedent. The $BaSO_4$ adsorbed serum has, however, been shown to be free of both antihemophilic globulin as well as plasma thromboplastin component.

The prothrombin utilization test, as modified by the addition of these specific corrective factors, has proved to be extremely useful in this laboratory as a differential diagnostic procedure in cases of AHG-, PTC-, PTA-, and combined AHG- and PTC-deficiencies.

TABLE III. PROTHROMBIN UTILIZATION TESTS

SAMPLE	ADDED FACTORS	% UTIL.
Patient's whole blood	None	< 5
Patient's whole blood	AHG concentrate	< 5
Patient's whole blood	Normal serum	14
Patient's whole blood	Adsorbed serum	11
Patient's whole blood	Protamine sulfate	82
Normal whole blood	None	>70
Patient's platelet-poor plasma	None	< 5
Patient's platelet-poor plasma	Normal platelets	< 5
Normal platelet-poor plasma	None	< 5
Normal platelet-poor plasma	Patient's platelets	>95
Normal platelet-poor plasma	Normal platelets	89

(17) Platelet thromboplastic factor(s). Results of this investigation are included in Table III. All blood and plasma specimens in this study were handled and processed in nonwettable containers. Needles were treated with Arquad 2C*; syringes, pipettes, and other glassware with Dri Film SC-87.† Lusteroid centrifuge tubes were employed during high-speed centrifugation. Sequestrene‡ was employed as an anticoagulant throughout the test. Twenty milliliter of normal blood was mixed gently with 0.6 ml. of 5 per cent Sequestrene solution and centrifuged at 1,000 r.p.m. for 20 minutes. The supernatant platelet-rich plasma was removed and recentrifuged at 3,000 r.p.m. for 20 minutes. The plasma thus obtained was centrifuged a third time for 30 minutes at 6,000 r.p.m. to assure complete removal of platelets and platelet fragments. Platelet-poor plasma prepared in this manner was employed as a source of all plasma thromboplastic factors in the testing of platelets. The platelet button obtained from the second centrifugation was washed twice by resuspension in excess 0.9 per cent saline solution and centrifugation. Platelet-poor plasma and washed platelets were obtained from the patient in an identical manner. Finally, the platelets were suspended in about 3.0 ml. of saline solution, quick-frozen, thawed, and manually homogenized prior to use.

Tests and controls were conducted as follows:

TUBE	PLATELET-POOR PLASMA (ML.)		PLATELETS (ML.)		SALINE SOLUTION (ML.)
	NORMAL	PATIENT	NORMAL	PATIENT	
1	2.0	--	--	--	0.1
2	2.0	--	0.1	--	--
3	--	2.0	--	--	0.1
4	2.0	--	--	0.1	--
5	--	2.0	0.1	--	--

Each tube was recalcified with 0.1 ml. of 0.3 M. $CaCl_2$ solution, incubated for one hour at 37° C., and the residual prothrombin determined by the Owren technique. Controls 1 and 3 must show a marked prothrombin utilization defect (less than 5 per cent consumption), and Control 2 must show a good utilization (greater than 70 per cent consumption); otherwise the plasma and platelet reagents are unsuitable.

(18) Thromboplastin generation tests, Biggs and Douglas[24] and Duckert and associates.[25] The results of this study are summarized in Table IV.

*Di Coco dimethyl Ammonium chloride, 75 per cent active in isopropanol, Armour & Co., Chicago.

†General Electric Chemical Division, Pittsfield, Massachusetts.

‡Ethylenediamine tetraacetic acid, Alrose Chemical Co., Providence, Rhode Island.

(19) Platelet morphology. Platelets were studied by the direct stained-film technique, by phase microscopy, and by electron microscopy, but no abnormalities in morphology were detected.

(20) Capillary microscopy. The capillaries of the fingernail bed were examined microscopically by the technique of Macfarlane,[26] but results were inconclusive.

TABLE IV. THROMBOPLASTIN GENERATION TESTS

REAGENTS			
PLASMA	SERUM	PLATELETS	RESULTS
Normal	Normal	Normal	Normal
Normal	Normal	Patient	Normal
Normal	Patient	Normal	Normal
Patient	Normal	Normal	Abnormal
Patient	Patient	Normal	Abnormal

In addition to the laboratory investigation of the propositus, his father, mother, and younger sister were also studied briefly. In all instances prothrombin, prothrombin utilization, and protamine titration were found to be normal.

DISCUSSION OF RESULTS

The laboratory investigation of the hemorrhagic defect in this patient, as summarized in Tables I through IV, showed no deficiency or abnormality in platelet count, bleeding time, prothrombin, proconvertin, proaccelerin, fibrinogen, fibrinolysin, vitamin C, platelet thromboplastic factor(s), platelet morphology, or thrombin clotting time. Certain tests performed in this case, however, were markedly abnormal: (1) whole blood clotting time was grossly prolonged; (2) Quick "prothrombin time" was abnormal; (3) Dalldorf test showed increased capillary fragility; (4) the antithrombin titer was elevated; (5) protamine titer was elevated; (6) heparin content was above normal; (7) prothrombin utilization and thromboplastin generation tests were markedly abnormal; and (8) a nonspecific test for anticoagulants was positive.

TABLE V. ANTITHROMBIN ACTIVITY

SAMPLE	ADDED FACTORS	ANTITHROMBIN (UNITS/ML.)
		76
Normal plasma	None	(56-110)
Patient's plasma	None	140
Normal plasma + 10 unit % heparin	None	128
Normal plasma + 10 unit % heparin	Protamine	0
Patient's plasma	Protamine	0

Initially, the poor prothrombin utilization with the prolonged Lee and White clotting time was quite suggestive of hemophilia or one of the "hemophilioid" diseases. Further testing, however, appeared to exclude such possibilities. The positive test for anticoagulants indicated that this patient's condition resulted from the presence of an abnormal coagulation inhibitor rather than from any deficiency condition. This postulate was strengthened

by the elevated antithrombin titer, the abnormal protamine titration, and especially by the elevated heparin content, as measured by the Freeman method. These observations are compatible only with a diagnosis of hyperheparinemia (that is, the presence of an excess of a heparin-like anticoagulant). The most striking confirmation was derived, however, from the prothrombin utilization and antithrombin tests. These tests demonstrated that protamine was capable of correcting the observed defect in each case, while such substances as antihemophilic globulin concentrate, normal serum, adsorbed serum, and normal platelets were unable to do so.

Certain other laboratory findings in this case appear worthy of note. The Quick "prothrombin time" was abnormal, even in the presence of normal concentrations of prothrombin, proconvertin, proaccelerin, and fibrinogen. Such an abnormality has previously been reported in hyperheparinemia by Bell.[9] This is in contrast to hemophilia, where the Quick test is typically normal. Such a finding strongly suggests that this heparin-like inhibitor acts as an antithromboplastin, which is in accord with the observations of Bell and those of Crissalli and Cotellessa. The results of the thromboplastin generation test and prothrombin utilization determination are entirely compatible with such a conclusion.

It is also evident that the coagulation inhibitor in this case acts as an antithrombin as well as an antithromboplastin. This was clearly demonstrated by the Owen-Bollman antithrombin determination. It was of considerable interest that the addition of 10 units per cent of U. S. P. heparin (sodium) to normal plasma produced a marked defect as measured by this method. Further, the addition of protamine sulfate to the patient's plasma and to this heparinized normal plasma completely neutralized all measureable antithrombin activity. The antithrombic activity of commercial heparin (sodium) and of naturally occurring heparinoid anticoagulants has been recognized by previous workers.

The thrombin clotting time has been recommended by Biggs and Macfarlane[19] as a means of detecting anticoagulants of the heparinoid type. In fact, the addition of commercial heparin to normal plasma does result in a marked prolongation of clotting time. It has also been suggested, when the thrombin clotting time is prolonged, that the corrective effect of protamine or toluidine blue would serve as confirmatory evidence of the presence of such an inhibitor. In this case no abnormality was detected by this method. This is in sharp contrast to the obviously elevated antithrombin titer as measured by the Owen-Bollman procedure. The reason for this apparent discrepancy is not evident from the existing information. However, these observations do suggest that more than a single type of heparinoid anticoagulant can and does occur in normal and pathologic conditions, and that the anticoagulant studied in this particular case is not identical with commercial beef heparin.

It should be noted that this patient demonstrated a gross abnormality in the Dalldorf test of capillary fragility. Platelet count and vitamin C con-

tent of the blood are generally regarded as the factors most important in assuring capillary integrity; yet this patient's abnormality was easily demonstrable in the presence of normal concentrations of both these constituents.

Since this patient was first diagnosed as having an excess heparinoid anticoagulant, he has been treated for approximately fourteen months with toluidine blue. The toluidine blue has been used for maintenance therapy as the oral preparation (Blutene). During this period the incidence of bleeding episodes has been markedly decreased, and the general clinical state of the patient has been significantly improved. The excellent response of the patient to the therapy represents, in itself, very convincing evidence confirming the diagnosis of hyperheparinemia.

The early onset of the hemorrhagic condition in this patient strongly suggests a congenital abnormality; however, the family pedigree reveals no bruising or bleeding tendencies; hence it gives no clues as to the mode of inheritance (if any) of this anomaly.

In certain instances of acquired antithromboplastinemia, it has been suggested that the anticoagulant was acquired or developed as a result of numerous previous blood transfusions. In this case it might be felt that the patient had hemophilia (or a related hemophilioid disease), and that the deficiency condition was merely masked by the anticoagulant, which had resulted from many transfusions. This concept, although plausible, is incompatible with the observations and findings of this study: namely, that the patient's prothrombin utilization defect was completely corrected in vitro by the addition of protamine and in vivo by toluidine blue. Such could hardly have resulted if a true deficiency of AHG, PTC, or PTA were associated with a circulating heparinoid anticoagulant.

One of the most interesting and perhaps the most significant finding in the laboratory investigation of this case was the gross elevation in blood heparin as measured by the Freeman method. Since this testing technique has been available only a short time, no information has been accumulated relative to the blood heparin levels in various pathologic states. Preliminary studies in this laboratory have amply confirmed Freeman's observations on the normal range of plasma heparin, namely, 4.5 to 18 units per cent, with the normal mean of about 10 units per cent. An investigation in this laboratory of a limited number of patients having hemorrhagic diathesis due to circulating anticoagulants has revealed none with a plasma heparin content comparable to that demonstrated in this case.

SUMMARY

The case report and laboratory investigation of a patient with a hemorrhagic diathesis due to a circulating anticoagulant have been presented. The most significant findings were:

(1) The early onset of hemorrhagic episodes suggested that the coagulation abnormality was congenital in nature.

(2) The family pedigree was completely lacking in any prior history of this bleeding tendency.

(3) Clinically, the patient's bleeding symptoms were indistinguishable from those of hemophilia. Subcutaneous hematomas, excessive bruising, and deep tissue hemorrhage with muscle and joint involvement were prominent features in this patient.

(4) Laboratory investigations excluded such conditions as afibrinogenemia, hypoprothrombinemia, hypoproconvertinemia, proaccelerin deficiency, hemophilia, PTC or PTA deficiency, and various other abnormalities which might simulate hyperheparinemia.

(5) The elevation of plasma antithrombin, protamine titer, and plasma heparin content were significant in differential diagnosis in this case. The antithromboplastic nature of the anticoagulant was easily demonstrated in the prothrombin utilization test and the thromboplastin generation determination. The correction of this antithromboplastic, as well as the antithrombic activity, by means of protamine sulfate was observed.

(6) Oral toluidine blue therapy during a period of fourteen months proved beneficial in controlling the hemorrhagic condition.

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THE RELATIONSHIP BETWEEN THE ALBUMIN CONCENTRATION OF SERUM AND ITS DYE-BINDING CAPACITY

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QUALITATIVE changes in serum albumin during the course of disease have been suspected, but not satisfactorily demonstrated.[1] As evidence for such changes, recent workers have cited reduction in the "dye-binding capacity" of the albumin of pathologic serum.[2-5] This dye-binding capacity of albumin for certain acid dyes is equal to the amount of dye bound by serum divided by the concentration of albumin, the only normal serum protein that binds these dyes.*

Conditions in which reduction in dye-binding ability have been reported include cancer, rheumatoid arthritis, chronic kidney and liver disease, infections,[3] and pregnancy.[5] On the other hand, some authors have found no alteration in dye-binding during the course of disease.[6, 7]

The present investigation of this moot problem not only showed that dye-binding is normal during diseased states; it also revealed the basis for the conflicting conclusions drawn by different workers from similar experiments. The ratio of dye bound to the serum albumin concentration, the so-called "dye-binding capacity" (referred to hereafter as the dye-binding ratio) was found to vary directly with the albumin concentration. Therefore, changes in this ratio do not necessarily indicate qualitative changes in albumin, but usually only reflect changes in albumin concentration.

MATERIALS AND METHODS

Blood was drawn from resting patients in the fasting state. The amount of recrystallized phenolsulfonphthalein (PSP) bound by 1 ml. of serum was determined by equilibrium dialysis following the technique of Huggins.[3] The only deviation from this technique was in the speed and type of motion of the shaking machine, but equilibrium was reached well before the end of the 48-hour shaking period. The amount of PSP bound by the dialysis bag in the absence of serum was about 15 micrograms.

Albumin was determined by the immunological method of Kunkel and Ward,[8] or by micro-Kjeldahl or biuret method after Howe fractionation as described by Weichselbaum.[9] Nonprotein nitrogen, determined by nesslerization, was subtracted from the nitrogen found by micro-Kjeldahl determination.

Albumin solutions were prepared from powdered bovine albumin† (Fraction V) which contained small amounts of sodium citrate, and from concentrated solutions of human

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*The nature of the bond between proteins and dyes has been studied intensively by Scatchard[16] and Klotz.[17]

†Armour Laboratories.

albumin* containing 0.02M sodium caprylate and 0.02M sodium acetyltryptophane as stabilizing agents. M/15 phosphate buffer, pH 7.4, was used as the diluent.

RESULTS

The amount of PSP bound by 56 sera of 51 patients with various diseases and by 15 sera of 13 normal subjects was measured and compared with the albumin content of the sera (Table I). There was a striking correlation between these two sets of values except in the case of jaundiced sera. The coefficient of correlation (r) for 61 nonjaundiced sera in which albumin was

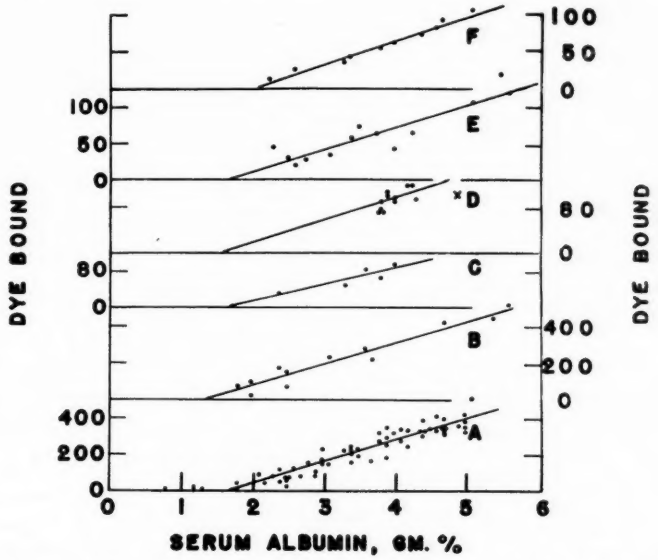

Fig. 1.—Dye-binding by serum: Regression lines calculated from the data of different authors.

GRAPH	AUTHOR	DYE	UNITS	BINDING METHOD	ALBUMIN METHOD	r*	SUBJECTS
F	Bennhold[2]	Naphthol yellow	% bound $\times 10^3$	Gelatin diffusion	?	0.99	Patients
E	Bennhold[6]	Azorubin	10^{-5} moles per c.c.	Chromatography	Howe fractionation	0.92	Patients
D	Miller[5]	PSP	gamma per c.c.	Equilibrium dialysis	Electrophoresis	0.54	Patients
C	Westphal[13, 14]	Azorubin	10^{-5} moles per c.c.	Chromatography	Electrophoresis	0.91	Pregnant women Rats and rabbits
B	This series	PSP	gamma per c.c.	Equilibrium dialysis	Immunological	0.98	
A	This series	PSP	gamma per c.c.	Equilibrium dialysis	Howe fractionation	0.94	Patients

*r is the correlation coefficient. In Graph *D*, x indicates a possibly aberrant value not included in the calculation of the regression line. Note that all graphs intercept the x-axis between 1.4 and 2 Gm. per cent.

determined after Howe fractionation was 0.94. The coefficient of correlation for 12 sera in which albumin was determined by the immunological method was 0.98. On graphic representation of the data obtained with both methods, all values, excluding those for jaundiced sera, fell closely along straight lines which intercepted the x-axis (albumin concentration) to the right of the

*E. R. Squibb & Sons.

TABLE I. BINDING OF PSP BY NORMAL AND PATHOLOGICAL SERA

NO.	DIAGNOSIS	ALBUMIN (GM. %) HOWE FRACTION. BIURET	MICROKJ.	IMM.†	DYE BOUND, GAMMA	DYE-BINDING RATIO
1	Normal*		5.1	5.6	510	100
2	Normal*		5.0	4.7	420	84
3	Normal	5.0			380	76
4	Carcinoma of breast	5.0			355	71
5	Normal	5.0			325	65
6	Normal*	4.9			360	73
7	Normal	4.7			395	84
8	Normal	4.7			345	73
9	Normal	4.7			345	73
10	Normal	4.7			335	71
11	Normal	4.7			320	68
12	Cirrhosis		4.6	5.4	410	89
13	Normal	4.6			335	73
14	Normal	4.5			345	77
15	Normal	4.4			390	89
16	Normal	4.4			335	76
17	Malignant hypertension	4.4			330	75
18	Duodenal ulcer	4.4			300	68
19	Normal	4.2			340	81
20	Hyperparathyroidism, coronary sclerosis	4.2			245	58
21	Filariasis, ankylostomiasis	4.1			340	83
22	Rheumatoid arthritis	4.1			285	70
23	Hyperthroidism	4.0			320	80
24	Pregnancy*	3.9			355	91
25	Duodenal ulcer, pyloric stenosis	3.9			295	76
26	Rheumatic heart disease, CHF	3.9			250	64
27	Diabetes mellitus, generalized arteriosclerosis	3.9			180	46
28	Carcinoma of bronchus with metastases	3.8			315	83
29	Pregnancy*	3.8			275	72
30	Lymphatic leukemia	3.7			165	45
31	Cirrhosis			3.6	280	78
32	Cirrhosis		3.5	3.1	230	66
33	Asthma, cor pulmonale, CHF, glomerulonephritis	3.5			195	56
34	Typhoid	3.4			245	72
35	Cirrhosis		3.4	3.7	220	65
36	Rheumatic fever	3.4			205	60
37	Hyperthyroidism, coronary sclerosis, CHF	3.4			155	46
38	Esophageal ulcer, cardiospasm	3.3			255	68
39	Rheumatic heart disease, CHF	3.1			145	47
40	Pernicious anemia	3.0			230	77
41	Cirrhosis		3.0	2.4	170	57
42	Generalized tuberculosis and arteriosclerosis	3.0			150	50
43	Carcinoma of ovary with metastases	2.9			105	36
44	Carcinoma of thyroid with metastases	2.9			90	31
45	Hyperthyroidism, cirrhosis		2.8	2.5	150	54
46	Diffuse vascular disease*	2.7			80	30
47	Paraganglioma, retroperitoneal	2.6			125	48
48	Cirrhosis			2.5	70	28
49	Hypothyroidism, cirrhosis	2.5			70	28
50	Carcinoma of stomach with metastases	2.5			65	26
51	Subacute glomerulonephritis*	2.5			65	26
52	Cirrhosis		2.5	2.0	20	8
53	Bronchopneumonia, malnutrition	2.4			110	46
54	Diffuse vascular disease*	2.4			55	23
55	Carcinoma of ovary with metastases	2.3			85	37
56	Subacute glomerulonephritis*	2.2			45	20

TABLE I—CONT'D

57	Rheumatic heart disease, CHF	2.1			95	45
58	Cirrhosis*		1.8	1.8	40	22
59	Cirrhosis*		1.8	1.4	10	6
60	Nephrotic syndrome*	1.3			0	0
61	Nephrotic syndrome	1.2			15	13
62	Nephrotic syndrome	1.2			0	0
63	Nephrotic syndrome*	0.8			0	0
	Jaundiced Patients					
64	Rheumatic heart disease, CHF, serum hepatitis	3.7			100	27
65	Rheumatic heart disease, CHF, cardiac cirrhosis	3.5			120	34
66	Rheumatic heart disease, CHF, cardiac cirrhosis	3.3			95	29
67	Infectious hepatitis	3.2			15	5
68	Carcinoma of breast with metastases	3.2			0	0
69	Cirrhosis		3.0	2.8	0	0
70	Henoch-Schönlein purpura	2.7			45	17
71	Empyema of gall bladder, cholangiolitic hepatitis	2.4			10	4

*Subject in whom two specimens of serum were examined at different times.
†Immunological.
CHF indicates congestive heart failure. The dye-binding ratio is the gamma of PSP bound per cubic centimeter of serum divided by the albumin concentration in Gm. per cent. The albumin value for Howe fractionation is used in the calculation where available.

origin (Fig. 1, *A* and *B*). The regression line for bound dye on the albumin concentration calculated from the values found after Howe fractionation, intercepted the x-axis at the 1.65 Gm. per cent mark, and that for the immunological albumin values at the 1.4 Gm. per cent mark.

From these regression lines it would appear that sera with these critical albumin concentrations, or lower, do not bind any dye. This was confirmed by the finding that three sera from two cases of nephrosis with 0.8, 1.2, and 1.3 Gm. per cent of albumin, respectively (Howe fractionation), did not bind any measurable amount of dye. (These three values are indicated in Fig. 1, *A*, but were not used in calculating the regression line.)

The formula for these regression lines is $y' = a(x\text{-}x_0)$, where y' is the predicted amount of dye bound, x the given total serum albumin concentration, x_0 the critical albumin concentration and a, a constant representing the slope of the line. For values falling along such regression lines which do not intercept the x-axis at the origin, y/x, the dye-binding ratio, falls progressively as x (the albumin concentration) diminishes (Table I). Therefore, the dye-binding ratio is at least in part an expression of the albumin concentration. A low ratio thus reflects a low albumin concentration, and does not necessarily indicate abnormality of the albumin. An abnormal serum dye-binding ratio would be one lower than that of sera of equal albumin content. Such abnormally low ratios were found in the case of jaundiced sera (Table I, Jaundiced Patients). Presumably, excess serum bilirubin competes with added artificial dye for binding sites on the albumin.

Unlike whole sera of varying albumin content, progressive saline dilutions of normal serum gave values which on graphing fell along a line intercepting the x-axis at the origin (Fig. 2, *A* and *B*). Similar results were found for solu-

tions of human and bovine albumin (Fig. 2, *D* and *C*). For such solutions which give more or less straight-line graphs passing through the origin, the dye-binding ratio is approximately the same at all albumin concentrations. The amount of PSP bound by solutions of human and bovine albumin was found to be less than that bound by dilutions of human serum (Fig. 2). This was probably due to the presence of added sodium caprylate in the stock solution of concentrated human albumin, and traces of citrate contaminating the commercial preparation of bovine albumin.[10] Since fatty acids are also bound by albumin, they reduce the amount of dye bound by competing for binding sites.[11, 12] Citrate interferes with the binding of ions by albumin,[10] and probably of dyes as well.

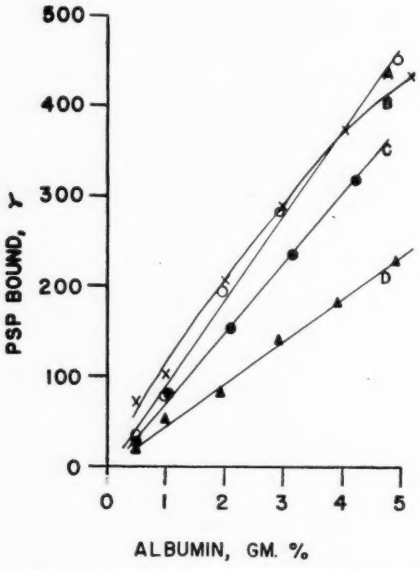

Fig. 2.—Binding of PSP by dilutions of whole serum and of serum albumin. *A* and *B*, Dilutions of normal human serum. *C*, Dilutions of bovine albumin containing sodium citrate. *D*, Dilutions of human albumin containing sodium caprylate and acetyltryptophane.

Preliminary results of studies of dye binding by specimens of ascitic, pleural, and synovial effusion fluid show values for dye binding that correspond well with those obtained for serum. Therefore these fluids, like serum, do not appear to bind dye when their albumin content is less than about 1.5 Gm. per cent.

DISCUSSION

The difference in dye-binding behavior between whole serum as opposed to dilutions of serum or solutions of albumin is at the heart of the dye-binding problem. Whole serum below a critical albumin concentration of about 1.5 Gm. per cent does not bind dye to a significant extent under the conditions used in this study. Serum whose albumin content is greater than this critical concentration binds dye only in proportion to that amount of albumin which is in excess of the critical concentration. On the other hand, albumin solutions

and saline dilutions of serum bind dye in direct proportion to their total albumin content. As shown above, this results in dye-binding ratios for whole serum which decrease with diminution of the albumin concentration, while the ratios for albumin solutions and dilutions of whole serum do not vary significantly with albumin content.

It is possible that a certain number of binding sites on the albumin of whole serum are occupied by various substances such as naturally occurring pigments (bilirubin, carotene), vitamins, bile acids, fatty acids, and others. This would explain the finding that an apparent fraction of total serum albumin, so far observed to vary only in the case of jaundice, does not bind added dye. This critical concentration of albumin may represent an amount of albumin whose total binding sites equal in number those bound to these naturally occurring substances. It may also reflect the presence of an albumin of very low binding capacity present in the serum of both patients and normal subjects.

In the process of isolating albumin from serum, substances occupying binding sites may be removed, allowing all the suitable binding sites to bind dye. This would explain Huggins'[3] finding that the albumin of sera with low binding ratios gave higher ratios after isolation. In the case of normal serum as well, he found that the isolated albumin bound more dye than the albumin in the original serum.

In Westphal's[4] report in which the "specific ABC" (azorubin-binding capacity) of pathologic human serum was found to be abnormally low, no albumin levels are tabulated, but it is possible to approximate roughly the average serum albumin of each of his groups of patients. The "observed ABC's" correlate well with these approximated ranges of albumin concentration except for unusually low binding in jaundiced patients and high binding in one case of myeloma. In the latter condition abnormal globulins that bind dye have been reported.[3]

In Westphal's[13] later studies of dye binding, involving animals subjected to tourniquet shock and carbon tetrachloride injection, the average albumin level for each of three groups of rats correlated fairly well with the average amount of dye bound ($r = 0.88$). Interestingly enough, the values for normal rabbits and those subjected to tourniquet shock[14] fell close to the line graphically relating dye bound to albumin concentration for rats (Fig. 1, *C*). The regression line calculated for the combined data of both rats and rabbits intercepts the x-axis at 1.6 Gm. per cent. This suggests that under certain conditions the albumins of these two species may bind PSP to the same extent, and that their sera have the same critical albumin concentration below which no dye binding occurs in human serum.

Huggins' group[5] reported that the serum of pregnant women binds PSP defectively. Their data give a regression line intercepting the x-axis at 1.6 Gm. per cent or at 0.04 Gm. per cent, depending on whether or not a single possibly aberrant value is excluded from the calculations (Fig. 1, *D*). In the former case the regression line indicates a critical concentration of albumin below which no dye is bound. However, if, as in the second case, the x-axis is

intercepted almost at the origin, then the serum of pregnant women binds dye like dilutions of normal serum. This would be logical since the low albumin levels in pregnancy are due to hydremia, a physiological dilution, and do not signify absolute hypoalbuminemia.

In Bennhold's[2] earlier work the wide scatter of the albumin levels of sera binding equal amounts of naphthol yellow suggested that disease results in variations in dye-binding capacity. However, the average of the albumin values for the various levels of dye bound shown in Bennhold's graph correlates almost perfectly with the amount of dye bound ($r = 0.99$). The regression line (calculated from these averages) indicates no dye binding below 2 Gm. per cent of albumin (Fig. 1, *F*). This critical concentration is higher than that of other series, probably because so many jaundiced cases were included.

From a later study of 11 patients and two normal subjects, Bennhold[6] concluded that disease does not cause abnormality in dye binding. He also noted the correlation between bound dye and albumin concentration and that little or no dye is bound by serum of very low albumin content. The regression line calculated from his data indicates no binding below 1.6 Gm. per cent of albumin (Howe fractionation) (Fig. 1, *E*).

Both Robinson and Hogden[7] and Smith and Smith[15] also concluded that dye-binding is normal in disease. The first workers studied the changes in optical density occurring when albumin binds PSP, and the latter workers used ultrafiltration of mixtures of serum and PSP. In both cases evidence was found for binding of dye by serum of very low albumin concentration, as opposed to the findings presented above and to those of Bennhold.[6] Further work is in progress to clarify this discrepancy which is probably due to the differences in technique.

The conclusions of this study are supported by the introduction of new methods[18, 19] for determining serum albumin based on demonstrations of the direct relationship between dye-binding and albumin concentration. These procedures measure the hypoalbuminemia which occurs only in pathologic serum, and therefore furnish additional evidence of the normality of dye-binding in diseased states.

SUMMARY AND CONCLUSIONS

The amount of dye bound by the serum of patients and normal subjects correlates closely with the serum albumin concentration, except when jaundice is present. Analysis of reports in the literature in which albumin levels are expressed, or can be estimated, confirms this finding.

From the data of this investigation as well as from those of several other reports, it appears that whole serum with less than a critical concentration of approximately 1.5 Gm. per cent of albumin does not bind dye to a significant degree. As a mathematical consequence, the ratio of dye bound to albumin concentration falls progressively with decrease in albumin concentration. Therefore, this ratio, often assumed to represent dye-binding capacity, does not usually indicate reduced or abnormal ability of albumin to bind dyes.

Solutions of albumin or saline dilutions of serum bind dye in proportion to their full albumin content. For these solutions the dye-binding ratio is approximately the same at all concentrations of albumin.

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A COMPARISON OF THE DEUTERIUM OXIDE AND ANTIPYRINE DILUTION METHODS FOR MEASURING TOTAL BODY WATER IN NORMAL AND HYDROPIC HUMAN SUBJECTS

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THE investigation reported here was undertaken to evaluate the antipyrine (ANP) and heavy water (D_2O) dilution methods for estimating total body water (TBW) in normal and hydropic subjects. The study was designed to determine (1) which method is the more accurate and reproducible in normal subjects; (2) possible limitations of the methods for use in the study of abnormal states; and (3) whether the methods could be adapted to the study of large groups of individuals.

The D_2O dilution method for the determination of TBW depends on the principle of isotope dilution.[1] The requirements for a perfect tracer for water are closely approximated by D_2O. D_2O forms an ideal solution with water.[2, 3] It has been shown[4] that D_2O administered intravenously to normal subjects equilibrates in all body fluids sampled (arterial blood, liver, gastric juice, venous blood, cisternal fluid, and urine) within two hours. The concentration remains the same for many hours, provided that there is no gross water intake. Constant venous concentrations are obtained within two hours following oral administration of D_2O.[5] D_2O is not known to be selectively utilized or excreted in the concentrations used.[5] No differences have been found in the deuterium to protium (ordinary hydrogen) ratio in biologic systems compared with the ratios found elsewhere in nature[6-8] within the limits of measuring sensitivity. A small correction factor to account for the deuterium initially bound to the organic constituents of the body will be discussed later.

The mass spectrometer has been used to determine deuterium in the water of all body fluids with a high degree of accuracy.[9] The falling drop method[10] gives accurate results with most body fluids except urine. The latter method has recently been modified[11] to give accurate results with urine also.

The antipyrine method, on the other hand, depends upon estimating the initial venous concentration of a substance (ANP) whose rate of disappearance in the body (following intravenous administration) is proportional to its concentration at a given time. It is rapidly metabolized, it is bound to proteins to an appreciable extent, and it equilibrates slowly with body fluid accumulations.[12]

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The chemical methods for the analysis of ANP do not yield as high recoveries or precision as do the analytic methods for determining D_2O. Nevertheless, the ANP method has yielded values for total body water which are comparable to values obtained by the D_2O method.

According to results of three comparative studies made on normal subjects, there is good agreement between the two methods, although the D_2O method was noted to give slightly greater TBW values. Soberman and associates[13] reported on eight adult males; Steele and co-workers[14] on six adult males; and Friis-Hansen and associates[15] on twenty children.

There have been two reports of similar comparisons on hydropic patients. One was by Soberman and co-workers[13] on seven subjects where it was found that there was a greater difference between the two methods than in the normal series. The other series was reported by Hurst and associates,[16] who studied twenty-eight edematous subjects and found that the D_2O spaces averaged 17 per cent greater than the ANP spaces.

MATERIALS AND METHODS

Subjects.—Thirty-three normal adult male subjects were studied. Some of them were enlisted Army personnel, and some were patients in the hospital awaiting minor elective surgery. Twelve male subjects with abnormal collections of fluid were also studied. They included six patients with ascites, four of whom were studied twice for a total of ten determinations, three with peripheral edema secondary to cardiac decompensation, one of whom was studied twice, and two with nutritional edema.

Food and liquid were withheld from all the subjects for twelve hours preceding the experiment. They were allowed nothing to eat or drink during the period of the test except in the extended tests, where the sick patients were allowed minimum fluid and food intake after six hours.

Scope of Experiments.—Total body water was determined on all subjects, using D_2O in all cases, and ANP in all cases where direct comparisons between the two methods were made. In the edematous subjects, rates of equilibration of the two substances between the edema (or ascitic) fluid and the serum (and in some cases, the urine also) were compared.

All experiments were preceded by collecting two "blank" samples of fluids to be studied. All samples were accurately timed.

Method of Administering, Sampling, and Determination of D_2O.—

A. *Administration:* Deuterium oxide (99.5 per cent pure, pyrogen-free, made isotonic with 0.9 per cent sodium chloride) was injected intravenously (or intraperitoneally in some of the ascitic patients) in doses of approximately 0.9 Gm. per kilogram body weight. The syringe was weighed to the nearest 0.01 gram before and after injection to determine the amount administered. When the D_2O was administered orally, 99.5 per cent pure D_2O was used, the amount ingested being determined by weighing the glass containing the D_2O before and after drinking the water.

B. *Sampling:* In the normal group accurately timed blood samples for D_2O determinations were collected at two and one-half and four hours and, in some cases, at approximately five and six hours as well.

In the edematous subjects simultaneous blood, edema (or ascitic) fluid, and, in some cases, urine samples were collected at two-hour intervals up to six hours, and then at nine and twelve hours (all times accurately noted). On some occasions, where massive edema was involved, eighteen-and twenty-four-hour samples were also withdrawn.

Ten-milliliter samples of venous blood were collected. Peripheral edema fluid was collected using a large (17 gauge), multiholed needle according to the method of Burch.[17] The needle was inserted into the subcutaneous tissues and its hub capped until a collection was to be made. By exerting gentle pressure on the edematous part, 8 to 10 ml. of edema fluid was obtained in 30 to 60 seconds.

Ascitic fluid (5 to 10 ml.) was collected through an indwelling plastic tube which was left taped to the abdominal wall between collections.

Urine samples were collected at approximately the same times as were samples of body fluids. Early urine samples were collected, but were not used in the determination of TBW because of the varying initial concentrations of D_2O. Later samples (at four and six hours approximately) were used for TBW comparisons in normal subjects. In the hydropic subjects, still later samples were necessary in order to ensure that equilibrium had been reached.

Blood samples were immediately contrifuged after collection and the serum sealed in glass ampules at –15° C. until analyzed. The other fluids were stored in a similar manner until analyzed.

C. *Analysis:* The D_2O in all fluid samples except urine was determined by the falling-drop method of Schloerb and associates.[16] Recoveries of heavy water added to serum in the range of 1,000 to 2,000 parts per million averaged 99.0 ± 1.4 per cent. Urine samples were determined by a modification of the falling-drop method in which a preliminary oxidation was performed.[11] The recoveries from urine in this range were 99.6 ± 0.2 per cent.

The D_2O space was calculated by the following equation:

$$\text{Total body water (liters)} = \frac{\text{Weight (grams) of } D_2O \text{ administered}}{\text{Grams of } D_2O \text{ per liter}}.$$

An estimate of 0.4 per cent of the injected dose of heavy water has been used by Schloerb and co-workers[5] to account for that lost until the time of sampling. This estimate was not used in the previously given calculations because of its small size and its probable great variability (dependent upon environmental conditions).

Method of Administering, Sampling, and Determination of ANP.—

A. *Administration:* Antipyrine was administered at the same time as was the D_2O. For intravenous injection, 2 Gm. (in a 20 per cent solution) was injected. Oral administration was accomplished either (a) by dissolving a weighed amount of ANP (2 Gm.) in a glass of water and having the subject drink the solution, followed by another drink of plain water from the same glass in order to ensure that all the ANP was ingested; or (b) by administering a capsule containing a known amount of ANP (2 Gm.) with a glass of water. All weighings were made to the nearest milligram.

B. *Sampling:* In normal subjects, 10 ml. of blood was collected for D_2O and antipyrine analysis at four-, five-, and six-hour intervals following their administration. Sample collection for analysis was extended over a longer period of time in edematous subjects.

Antipyrine was analyzed by the method of Brodie and associates.[12] An Evelyn photometer was used, and recoveries of known quantities of ANP from serum by this method were 100.8 ± 2.2 per cent. The concentration of ANP at the time of injection was determined by extrapolation of the concentrations on a semilogarithmic plot to zero time. The method of least squares was used to determine the exact concentration at zero time.

RESULTS

Normal Subjects.—

A. *Comparison of D_2O spaces and ANP spaces:* Simultaneous D_2O and ANP space measurements were made in thirty-three subjects (Table I). The average D_2O volume for the group was 43.4 L. or 61.2 per cent of the body

weight, while the average ANP volume was 41.5 L. or 58.5 per cent of the body weight. The average difference between the spaces was 1.9 ± 2.8 L., and the average ratio of D_2O space to ANP space was 1.05 ± 0.07 ($p = 0.01$).

B. *Comparison of oral versus intravenous D_2O spaces:* A comparison of the spaces obtained from the oral and intravenous administration of D_2O was made in four individuals by alternating the methods in two consecutive determinations (Table II). The difference between the two methods averages 0.7 ± 4.2 per cent ($p > 0.99$) of the total body water as a percentage of the body weight.

C. *Equilibration of serum D_2O with urinary D_2O:* Table III shows that urinary D_2O equilibrates with serum D_2O within four hours provided that early urine samples following D_2O administration are discarded. A value of 1.00 ± 0.01 for the ratio of the four-hour urine sample to the five- (or six-) hour urine sample shows that the measurement of D_2O from urine sample to urine sample is reproducible.

TABLE I. COMPARISON OF DEUTERIUM (D_2O) AND ANTIPYRINE (ANP) SPACES IN NORMAL MALE SUBJECTS (INTRAVENOUS ADMINISTRATION)

SUBJECT	BODY WEIGHT (KG.)	D_2O SPACE (L.)	ANP SPACE (L.)	D_2O ANP RATIO	D_2O—ANP SPACE (L.)
D. P.	81.4	43.6	41.6	1.05	2.0
D. B.	99.9	59.5	61.0	0.98	−1.5
T. R.[1]	92.0	50.9	48.4	1.05	2.5
R. M.	79.3	46.6	42.3	1.10	4.3
O. J.	101.0	50.5	45.3	1.12	5.2
W. DeC.	63.5	36.4	38.0	0.96	−1.6
L. P.[1]	79.8	42.0	34.7	1.21	7.3
T. F.	73.2	44.8	42.3	1.06	2.5
L. P.[2]	79.5	41.1	36.5	1.13	4.6
T. R.[2]	91.3	46.1	44.1	1.05	2.0
M. G.	62.4	40.8	37.0	1.10	3.8
B. H.	80.5	47.2	40.7	1.16	6.5
L. C.	72.0	42.1	39.7	1.06	1.4
R. R.	68.4	36.1	34.1	1.06	2.0
D. R.	85.8	46.9	46.4	1.01	0.5
R. T.	81.3	46.8	46.8	1.00	0.7
G. H.	117.6	49.2	44.4	1.11	4.8
W. M.	71.7	44.8	41.8	1.07	3.0
F. S.*	80.4	46.8	45.6	1.03	1.2
B. K.*	78.7	46.5	49.5	0.94	−3.0
E. W. G.	67.0	42.7	41.6	1.02	1.1
W. F. K.	72.1	44.2	39.6	1.11	4.6
T. T. McD.	69.8	43.0	46.6	0.92	−3.6
S. S.	61.2	40.3	42.6	0.95	−2.3
A. W.	59.0	34.8	34.2	1.02	0.6
W. N.	74.0	40.6	39.9	1.01	0.7
W. N.	75.0	42.5	39.3	1.08	3.2
R. S.	58.3	34.4	32.9	1.04	1.5
R. S.	59.5	34.2	33.3	1.03	0.9
H. B. R.	68.0	43.3	35.7	1.22	7.6
H. B. R.	69.5	40.8	37.8	1.08	3.0
W. S.	79.3	41.0	43.3	0.95	−2.3
W. S.	81.4	43.8	41.9	1.04	1.9
Average	70.9	43.4	41.5	1.05	1.9
Standard deviation				±0.07	±2.8
					$p = 0.01$

*D_2O administered orally.

TABLE II. COMPARISON OF D_2O SPACES OBTAINED BY ORAL AND INTRAVENOUS ADMINISTRATION

SUBJECT	INTRAVENOUS		ORAL		PER CENT DIFFERENCE
	D_2O SPACE (L.)	PER CENT OF BODY WEIGHT	D_2O SPACE (L.)	PER CENT OF BODY WEIGHT	
H. S.*	43.0	45.9	42.6	45.9	−0.0
B. F.	49.3	55.1	51.7	56.3	−1.2
J. F. T.	46.5	66.4	42.5	61.7	4.7
W. S.[1]	41.0	52.2	42.5	61.2	−6.1 (avg.)
W. S.[2]	43.8	53.3	45.6	56.5	
Mean					−0.7± 4.2 $p > 0.99$

Similar low values for total body water as per cent of body weight have been reported for normal males.[21]

*Obese.

TABLE III. EQUILIBRATION OF SERUM D_2O WITH URINARY D_2O*

SUBJECT	D_2O SERUM-URINE RATIO AT FOUR HOURS	SERUM-URINE D_2O RATIO AT FIVE HOURS	RATIO OF FIRST URINE TO SECOND URINE
A. J.	1.00	1.02	1.01
R. A.	1.02	1.05	1.03
J. B.	1.01	1.02	1.01
S. M.	1.00	1.00	1.00
J. M.	1.02	1.02	0.99
F. K.	1.00	1.01	0.99
O. P.	1.00	0.99	0.99
B. M.	0.97	0.97	1.00
M. M.	1.01	0.98	0.99
X. P.	1.01	1.00	0.99
Average	1.01 ± 0.01	1.01 ± 0.02	1.00 ± 0.01

*In each case one or two urine samples were collected prior to the four-hour samples and discarded.

D. *Comparison of oral ANP versus D_2O spaces:* Antipyrine was administered orally to five subjects, one of them on two occasions, and the resulting total body water values were compared with simultaneously measured D_2O spaces (Table IV). The average difference between the spaces was 3.5 ± 2.1 L. The average ratio of the D_2O space to ANP space was 1.09 ± 0.05 ($p = 0.02$).

TABLE IV. COMPARISON OF D_2O SPACES WITH ORAL ANP SPACES IN NONEDEMATOUS MALE SUBJECTS

SUBJECT	D_2O		ANTIPYRINE SPACE (L.)	DEUTERIUM/ ANTIPYRINE RATIO	DEUTERIUM SPACE MINUS ANTIPYRINE SPACE (L.)
	LITERS	ROUTE OF ADMINISTRATION			
H. S.	43.0	I.V.	40.7	1.05	2.3
B. F.	49.3	I.V.	45.0	1.09	4.3
P. S.	37.4	P.O.	34.6	1.08	2.8
C. C.	36.0	P.O.	35.6	1.01	0.4
W. S.	42.5	P.O.	37.8	1.12	4.7
W. S.	45.6	P.O.	38.9	1.17	6.7
			Average	1.09 ± .05	3.5 ± 2.1 $p = < 0.02$

E. *Reproducibility of D_2O spaces versus reproducibility of ANP spaces:* Studies of reproducibility of D_2O spaces and ANP spaces were made on eleven subjects (Tables V and VI). During the interval of time elapsing between determinations there were small weight changes in many of the subjects. There-

fore, comparisons were based on TBW calculated as per cent of body weight. In ten of the eleven subjects, the change in D_2O spaces was in the direction of change in weight. In four of the six ANP reproducibility experiments the change in water volume so measured followed also the change in body weight.

In the case of D_2O, seven of the eleven subjects gave results reproducible within the error of the method. With ANP three of six subjects gave results reproducible within the analytic error.

Hydropic Patients.—

A. *Equilibration times:* In the subjects having clinically detectable abnormal collections of fluid, it was found (Table VII) that the time required for equilibration of both substances in all body fluids sampled was invariably prolonged. In all cases but one, D_2O reached equilibrium at some time between two and twelve hours, and in the single exception it had occurred prior to a twenty-four-hour sample. It was noted that serum and urine attained constant values at about the same time as the ascitic and edema fluid.

In only two of six determinations in patients with ascites did equilibration with ANP occur between the ascitic fluid and serum. In the three cases with peripheral edema, equilibrium with ANP between serum and edema fluid occurred only after eight hours.

B. *Total body water:* Average total body water values (D_2O spaces) for the hydropic patients was 67.2 per cent of the body weight (Table VII). Total body water (ANP spaces) were not reported because even in cases where equilibration between the various fluids did occur, it was not possible to extrapolate a single straight line back to zero time to get a value for the initial concentration of ANP.

DISCUSSION

Normal Subjects.—The average ratio of the D_2O to ANP spaces on thirty-three normal subjects reported here is 1.05, a value which is 3 per cent higher than that previously reported by Steele and associates[14] in six subjects and by Soberman and co-workers[13] on eight subjects. The value of 1.05 may be reduced to 1.03 by taking into account the fact that the D_2O spaces are probably high by an estimated 1 to 2 per cent due to the exchange of deuterium with rapidly exchanging hydrogen atoms in the organic constituents of the body.[15] The value of 1 to 2 per cent was obtained by estimating the protein, carbohydrate, and fat content of the body and calculating the amount of freely exchangeable hydrogens that are available for exchange.

Deuterium oxide is more versatile than is ANP in the variations which can be used in conducting the procedure. As can be seen from Table II, there is no significant difference between TBW, as per cent of body weight, measured after oral or intravenous administration of D_2O. Orally administered ANP gives results which are 50 per cent more at variance with the D_2O values (Table IV) than does intravenously administered ANP. Urinary D_2O concentrations (provided that early urine samples are collected and discarded) are equal to serum D_2O concentrations after five or six hours. Therefore, appropriately collected urine samples instead of serum samples may be used to determine

TABLE V. STUDIES OF REPRODUCIBILITY OF TOTAL BODY WATER MEASUREMENTS IN NONEDEMATOUS MALE SUBJECTS

SUBJECT	DAYS AFTER FIRST MEASUREMENT	WEIGHT (KG.)	CHANGE IN WT. (KG.)	DEUTERIUM OXIDE			ANTIPYRINE			DISCREPANCY† ANP DISCREPANCY D_2O
				LITERS	METHOD OF ADMINISTRATION	DIFFERENCE IN VOLUME (L.)	LITERS	METHOD OF ADMINISTRATION	DIFFERENCE IN VOLUME (L.)	
D. B.	0	99.7	—	59.5	I.V.	—	—	—	—	—
	14	99.4	−0.3	57.3	I.V.	−2.2	—	—	—	—
L. P.	0	79.4	—	42.0	I.V.	−0.9	34.7	I.V.	1.8	2.4
	20	79.3	−0.1	41.1	I.V.	—	36.5	I.V.	—	—
T. R.	0	91.3	—	50.9	I.V.	—	48.4	I.V.	—	—
	56*	91.1	−0.2	46.1	I.V.	−4.8	44.1	I.V.	−4.3	0.9
D. P.	0	81.2	—	43.6	P.O.	—	—	—	—	—
	3	81.1	−0.1	42.7	P.O.	−0.9	—	—	—	—
	20	82.2	1.0	46.5	I.V.	2.9	—	—	—	—
W. S.	0	79.3	—	41.0	I.V.	—	43.4	I.V.	—	—
	14	81.4	2.0	43.8	I.V.	2.8	41.9	I.V.	−1.5	5.1
	26	83.3	4.0	42.5	I.V.	1.5	37.8	P.O.	−5.6	3.8
	37	80.9	1.6	45.6	I.V.	4.6	38.9	P.O.	−4.5	2.0
J. T.	0	68.9	—	44.9	P.O.	—	38.9	I.V.	—	—
	9	70.0	1.1	46.5	I.V.	1.6	35.2	I.V.	−3.7	8.0
W. N.	0	74.0	—	40.6	I.V.	—	39.9	I.V.	—	—
	8	75.0	1.0	42.5	I.V.	1.9	43.2	I.V.	3.3	2.5
R. S.	0	58.3	—	34.2	I.V.	—	32.9	I.V.	—	—
	8	59.5	1.2	34.4	I.V.	0.2	33.3	I.V.	0.4	0.8
H. R.	0	68.0	—	43.3	I.V.	—	35.7	I.V.	—	—
	8	69.5	1.5	40.8	I.V.	−2.5	37.8	I.V.	2.1	0.2
H. S.	0	93.2	—	42.6	P.O.	—	—	—	—	—
	9	93.2	0.0	43.0	I.V.	0.4	—	—	—	—
B. F.	0	91.8	—	51.7	P.O.	—	—	—	—	—
	9	89.5	−2.3	49.3	I.V.	−2.4	—	—	—	—
									Average	2.8

*Prolonged heat wave occurred prior to second measurement.
†Discrepancy = difference between weight changes and total body water weight changes.

total body water. Oral administration of D_2O and urinary D_2O determinations therefore extend the usefulness of this method. It should be mentioned, however, that some caution should be observed when D_2O is administered orally. In one instance a patient was given D_2O orally and suddenly, after one hour and twenty minutes had elapsed, he vomited. A sample of emesis was found to contain D_2O in a concentration many times that obtained in serum, indicating that absorption had been retarded.

TABLE VI. STUDIES ON REPRODUCIBILITY OF TOTAL BODY WATER AS A PERCENTAGE OF TOTAL BODY WEIGHT

SUBJECT	D_2O SPACE (L.)	ANP SPACE (L.)	WEIGHT (KG.)	PER CENT OF BODY WEIGHT (AVERAGE) D_2O	PER CENT OF BODY WEIGHT (AVERAGE) ANP
D. B.	59.5	—	99.7	58.6—1.0*	—
	57.3	—	99.4		
L. P.	42.0	34.7	79.4	52.3—0.5*	44.8—2.6
	41.1	36.5	79.3		
T. R.	50.9	48.4	91.3	53.2—2.6	50.7—2.3
	46.1	44.1	91.1		
D. P.	43.6	—	81.2	54.6—2.2	—
	42.7	—	81.1		
	46.5	—	82.2		
J. T.	44.9	38.9	68.9	65.8—0.6*	53.4—3.3
	46.5	35.2	70.0		
W. N.	40.6	39.9	74.0	55.8—0.9*	55.7—1.8*
	42.5	43.2	75.0		
R. S.	34.2	32.9	58.3	58.2—0.4*	56.2—0.2*
	34.4	33.3	59.5		
H. R.	43.3	35.7	68.0	61.2—2.5	53.4—0.9*
	40.8	37.8	69.5		
W. S.	41.0	43.4	79.3	53.2—2.4	49.9—4.0
	43.8	41.9	81.4		
	42.5	37.8	83.3		
	45.6	38.9	80.9		
H. S.	42.6	—	93.2	45.9—0.2*	—
	43.0	—	93.2		
B. F.	51.7	—	91.8	55.7—0.6*	—
	49.3	—	89.5		

*Within experimental error of the method.

Hydropic States.—It is not surprising that time for equilibration of D_2O and ANP in the hydropic states should be much longer than in normal subjects. It was observed in this study that D_2O eventually reached an equilibrium concentration in all the body fluids sampled, most equilibrations occurring prior to eight hours. The slow attainment of equilibration in these hydropic subjects is an agreement with the work of Hurst and associates.[16] This slow attainment of equilibration is probably the result of slow mixing of the water within the ascitic and edematous depot and also of an apparent slower rate of exchange of water than occurs between water compartments in normal individuals.[19]

The fact that D_2O equilibrates in all body fluids in edematous subjects within a reasonable period of time (twelve hours, or in massively edematous subjects, within twenty-four hours) makes it possible to measure total body water in these subjects. An error of perhaps 1 per cent is probably involved in the measurement of total body water in these patients, due to the slight dilution of the deuterium by a water intake of around 500 c.c. and a similar gradual

TABLE VII. THE DEUTERIUM OXIDE SPACE IN EDEMATOUS SUBJECTS WITH A COMPARISON OF THE EQUILIBRATION OF DEUTERIUM OXIDE AND ANTIPYRINE* IN VARIOUS FLUID COMPARTMENTS

SUBJECT	DIAGNOSIS	ROUTE D_2O GIVEN	BODY WEIGHT (KG.)	D_2O		EQUILIBRATION TIME OF D_2O BETWEEN SERUM AND:			EQUILIBRATION TIME OF ANP BETWEEN SERUM AND:	
				LITERS	PER CENT BODY WEIGHT	EDEMA FLUID	ASCITIC FLUID	URINE	EDEMA FLUID	ASCITIC FLUID
R. W. M.[1]	Cirrhosis	I.V.	62.2	45.3	72.8		12-24°			17°
R. W. M.[2]	Cirrhosis	Oral	68.6	49.4	72.1		10°			"
S. T. K.[1]	Cirrhosis	Oral	59.3	41.6	70.0		6°			6°
S. T. K.[2]	Cirrhosis	I.P.	62.4	47.3	75.6		9°			"
J. R. D.[1]	Ht. fail.	I.V.	70.9	45.8	64.7	12-24°			11°	
J. R. D.[2]	Ht. fail.	I.V.	70.6	42.5	60.2	8°			8°	
J. L. O.[1]	Cirrhosis	Oral	63.0	35.9	57.0		6- 7°			"
J. L. O.[2]	Cirrhosis	I.P.	64.5	44.8	69.5		4- 5°			"
J. L.	Cirrhosis	Oral	72.1	44.0	61.0		2- 3°			"
F. J. W.	Ht. fail.	I.V.	90.6	65.3	72.1	12°		8°	8°	
C. F. K.	Ht. fail.	Oral	71.1	47.7	66.5	6°30′	6°30′	6°30′		
J. I. P.	Hypoprot.	I.V.	68.5	42.1	68.5	8°		8°		
F. J. H.	Hypoprot.	I.V.	44.0	32.5	73.9	8°		3°		
L. R. E.	Cirrhosis	Oral	62.7	36.4	58.0		4°20′	5°		
Average			66.5	44.3	67.2					

*Antipyrine administered intravenously.

"———" Equilibrium was not reached in these cases in a 24-hour period.

loss via the urine. Since urinary D_2O and serum D_2O concentrations are equivalent by the end of eight hours in the edematous subjects studied here, the use of urinary D_2O to measure total body water following equilibration in the rest of the body water compartments is permitted. However, it is recommended that consecutive urine samples be analyzed to ensure that equilibration has occurred.

The behavior of ANP in hydropic states, on the other hand, does not qualify it for use in the study of total body water in these states. Hurst and co-workers[16] presented evidence to show that equilibration did not occur in most of their cases which had abnormal collections of fluid. An earlier study in this laboratory[20] indicated that ANP did not equilibrate in eight hours in seven subjects with cirrhosis and ascites or in nine subjects with peripheral edema. In this study, in only two of six determinations in patients with ascites did equilibration with ANP occur between the body fluids sampled in twenty-four hours. The three cases with peripheral edema included in this study in which ANP was used reached an equilibrium for this substance between the serum and edema fluid after eight hours. Even though equilibrium concentrations of ANP were obtained in some of these cases, it was impossible to extrapolate a single straight line back to zero time in order to obtain an initial ANP concentration; therefore, values for total body water could not be determined.

SUMMARY

The deuterium oxide and antipyrine spaces were studied in a large group of normal adult males. It was found that the D_2O spaces were consistently and significantly larger than the antipyrine spaces.

It was found that deuterium oxide could be used to measure total body water either by oral or intravenous administration with equally reliable results.

In edematous subjects D_2O was found to equilibrate in all body fluids so that it could be used to determine total body water in such subjects. Antipyrine, on the other hand, often failed to equilibrate within twenty-four hours, and it was concluded that its dilution was not a reliable measure of total body water in edematous states.

The antipyrine used in these studies was generously supplied by the Eli Lilly Company, Indianapolis, Indiana, and the deuterium oxide was purchased from the Stuart Oxygen Company of San Francisco, California. The deuterium oxide was prepared for intravenous use by Abbott Laboratories, North Chicago, Illinois.

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THE USE OF URINARY DEUTERIUM OXIDE CONCENTRATIONS IN A SIMPLE METHOD FOR MEASURING TOTAL BODY WATER

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THE determination of total body water by isotope dilution techniques has undergone extensive study in recent years. Deuterium oxide has been found to be an ideal substance for this purpose, since it is distributed uniformly throughout the body fluids within a relatively short time.[1, 2] The method generally employed has been that of analyzing venous serum samples which are drawn following the equilibration of an intravenously injected quantity of heavy water (D_2O). However, it has also been determined that equilibration of ingested D_2O follows the same pattern as intravenously administered D_2O.[1, 3, 4] In situations where multiple venipunctures would not be feasible or desirable, total body water could be measured by the oral administration of D_2O if urine samples, appropriately timed and collected, could be analyzed accurately.

Since D_2O is uniformly distributed throughout the body fluid, it was expected that its concentration in the urine following equilibration would be the same as in the blood serum from which it is derived, and in other body fluids. Hurst, Schemm, and Vogel[3] have indicated that this is so.

It was also of interest to determine whether this method could be extended to include ill patients and edematous subjects.

Because urine contains oxidizable organic impurities which cannot be removed by the simple vacuum distillation technique, the method of Schloerb, Friis-Hansen, Edelman, Sheldon, and Moore[5] for D_2O determinations cannot be directly applied to urine samples. However, through a modification of the method of Keston, Rittenberg, and Schoenheimer[6] for the purification of water samples obtained from the combustion of organic compounds, the objection to the use of urine samples was surmounted. This modification was developed by one of us (I. L. F.) and is described later. It then became possible to show that D_2O administered orally and intravenously in the same subject would measure the same values for total body water, and that identical concentrations of D_2O could be obtained from corresponding urine and serum samples.

METHODS AND MATERIALS

Preparation, Administration, and Collection of Deuterium Oxide.—All subjects were in a basal state, having ingested nothing during the twelve

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hours prior to the procedure. Blank urine and blood samples were obtained from each subject at the beginning. The subjects refrained from eating or drinking during the procedure. Heavy water (99.5 per cent D_2O) weighed to the nearest 0.01 Gm. was administered orally to four "normal" male subjects. The amount of D_2O given was approximately 0.9 Gm. per kilogram of body weight, depending on the body build, the leaner subjects receiving a slightly larger amount proportionately. Blood samples were drawn at four and at five hours after D_2O ingestion. It has been shown[1] that orally administered D_2O shows a constant serum level from two to eight hours following ingestion. The same four subjects were studied again, the heavy water being injected intravenously. Thirteen "normal" male subjects were given D_2O (prepared as isotonic saline solution, pyrogen-free) intravenously, the syringe being weighed to the nearest 0.01 gram before and after injection. Two venous samples were collected between three and six hours. Serum has been shown to maintain constant D_2O concentration from two to six hours following the intravenous administration of D_2O.[1] The subject emptied his bladder at three hours, and this sample was discarded, since initial residual water in the bladder would give erroneous values for the D_2O concentration. Voided urine specimens were collected at hourly intervals thereafter. Therefore, the interval collections were approximately third- and fourth- or fourth- and fifth-hour samples. These are approximate hours.

In disease states where there is a derangement in water metabolism and water distribution, longer periods of time are likely to be required for the equilibration of D_2O. Therefore, in these states, blood, urine, and edema fluid samples were taken at frequent intervals up to twenty-four hours. Minimum food and water were permitted after the first six hours of the procedure.

The blood samples were immediately centrifuged, the serum placed in ampules, sealed, and frozen until analyzed. The urine samples were sealed in ampules and frozen until ready for analysis.

Analysis of Samples for D_2O.—The serum samples were analyzed for their D_2O content by a falling-drop method in a constant temperature bath according to the method of Schloerb and associates.[5] Recoveries of heavy water added to serum in the range of 1,000 to 2,000 parts per million averaged 99.9 ± 1.4 per cent.

Approximately 1 ml. of urine sample was placed in the U tube of the purification train described by Schloerb and co-workers.[5] The sample was frozen by immersion in a dry ice and alcohol mixture and vacuum distilled into a trapped "in" tube for one hour. The "in" tube contained a few crystals of potassium permanganate (Mallinckrodt, U.S.P. grade) and anhydrous sodium carbonate (Mallinckrodt, analytic reagent grade). After the distillation was completed, the "in" tube was gently warmed with water at about 40° to 50° C. to melt the sample of water in the tube. The tube was removed from the train, capped, and placed in a steam bath for one hour to reflux the sample and the oxidative mixture. Soon after the sample had been placed in the steam bath, the pressure which had developed in the tube was released by uncapping the tube momentarily.

After the alkaline permanganate oxidation had proceeded for one hour, the tube was cooled slowly and replaced on the train, frozen with the dry ice and alcohol mixture, and the water was distilled into a second "in" tube. The procedure followed thereafter was exactly the same as that described by Schloerb and associates.[5] Recoveries of D_2O added to urine are 99.6 ± 0.02 per cent by this method.

Calculation of Total Body Water.—The volume of total body water was calculated from the following formula:

$$\text{Total body water (liters)} = \frac{\text{Actual weight of } D_2O \text{ administered (grams)}}{\text{Grams of } D_2O \text{ per liter}}.$$

An estimated 0.4 per cent of the injected dose of heavy water has been used by Schloerb and co-workers[1] to account for the heavy water excreted up until the time of sampling. This estimate was not used in our calculations because of the small value, and its probable variation.

RESULTS AND DISCUSSION

Both urine samples for each subject were analyzed in order to be certain that equilibration had occurred. These samples were not statistically different from each other, the differences falling within the analytic error of the method. Table I (a) shows the serum and urine deuterium oxide ratios for 13 normal subjects. It can be seen that the analyses of serum and urine samples give identical results within the error of the method. It is apparent that a single urine sample is sufficient for the determination. Table I (b) is a comparison of total body water values for the same 13 subjects obtained by both methods. It can be seen that the results are in excellent agreement.

TABLE I(A). SERUM AND URINE DEUTERIUM OXIDE RATIOS IN NORMAL MALE SUBJECTS

SUBJECT	AVERAGE SERUM / AVERAGE URINE	FIRST URINE / SECOND URINE	FIRST SERUM / FIRST URINE	SECOND SERUM / SECOND URINE
G. H.	0.991	--	0.995	--
W. M.	0.990	0.999	0.998	0.980
W. H.	0.999	--	--	1.009
T. R.[1]	1.015	0.999	1.008	1.022
T. R.[2]	1.019	1.049	1.010	1.029
L. P.[1]	1.023	0.993	1.028	1.017
L. P.[2]	1.009	1.027	0.999	1.020
R. H.	0.979	0.994	0.993	0.966
L. C.	1.017	0.997	1.018	1.016
D. B.	1.014	1.017	0.995	1.033
R. M.	1.004	--	1.004	--
O. J.	1.008	0.988	1.025	0.991
T. F.	0.989	0.986	0.998	0.980
Average	1.004	1.005	1.006	1.006
S. D.	0.026	0.018	0.010	0.014

TABLE I(B). A COMPARISON OF TOTAL BODY WATER MEASUREMENTS BY THE SERUM AND URINE D_2O METHODS IN NORMAL MALE SUBJECTS

SUBJECT	TOTAL BODY WATER—URINE (L.)	TOTAL BODY WATER—SERUM (L.)	RATIO SERUM/URINE
G. H.	49.1	49.2	1.002
W. M.	43.9	44.8	1.021
W. H.	47.2	47.2	1.000
T. R.[1]	51.9	50.9	0.981
T. R.[2]	47.0	46.1	0.981
L. P.[1]	43.0	42.0	0.978
L. P.[2]	41.6	41.1	0.988
R. H.	35.4	36.1	1.020
L. C.	42.8	42.1	0.984
D. B.	60.3	59.5	0.987
R. M.	46.0	46.6	1.013
O. J.	50.8	50.5	0.994
T. F.	44.2	44.8	1.014
Average			0.997
S. D.			0.020

During the period of equilibration some of the administered D_2O is excreted in the urine, and it must also be assumed that some is lost through expired water vapor and transpired from the skin. Under the conditions of our study it was shown that the amount lost in the urine was negligible. The urine formed during the equilibrium period was analyzed for D_2O content in seven subjects, two of whom were hospitalized patients. The results are shown in Table II. The amount lost in the urine during the first two to three hours during which equilibration is taking place amounted to 0.16 per cent of the injected amount. This corresponds to a volume of approximately 60 ml. in a man whose total body water is 40 L. While we have not yet determined the insensible loss of D_2O, if it is assumed that it is about equal to the urinary loss, the amount of D_2O lost from the body during the period of equilibration is negligible.

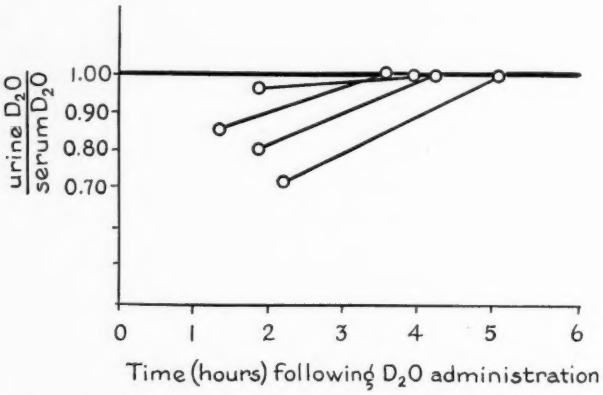

Fig. 1.—Approximate time of equilibration of orally ingested D_2O with body water.

In Fig. 1 ratios of urine D_2O to equilibrium serum D_2O in normal subjects are presented before and after equilibrium has occurred between serum and urine. It can be seen that early urine samples are dilute with respect to the serum D_2O concentration. However, second urine samples (collected three to six hours following D_2O administration) yield concentrations of D_2O equal to that of serum, well within the limits of experimental error.

Table III shows the lengths of time required for equilibration (ratio of serum to urine $D_2O = 1.00 \pm 0.02$) to occur between serum and urine D_2O in seven edematous subjects and two other subjects with severe metabolic disorders. It is important to note that considerable time intervals are required for D_2O equilibration to occur between urine and serum in edematous subjects. The error involved in the total body water measurement in these patients in probably no greater than 1 per cent. The error would be caused by a gradual loss of water via the urine (approximately 200 to 500 c.c.) and a slight dilution of the deuterium-containing total body water by the ingestion of approximately the same quantity of ordinary water. Because of this fact, it is advisable to take several urine samples for analysis (six to twelve hours following administration to D_2O) to ensure that equilibration has been reached.

TABLE II. D_2O LOSS VIA URINE DURING EQUILIBRATION PERIOD (TO THREE HOURS)

SUBJECT	D_2O LOST VIA URINE (GM.)	PER CENT OF ADMINISTERED D_2O LOST VIA URINE
T. R.	0.157	0.16
L. P.	0.124	0.16
R. M.	0.153	0.21
O. J.	0.097	0.12
T. F.	0.060	0.07
R. K. W.*	0.133	0.16
J. R.†	0.171	0.21
		Average 0.16
		This corresponds to about 60 c.c. loss in a total volume of 40 L.

*Cushing's syndrome.
†Cardiac decompensation with leg edema.

TABLE III. COMPARISON OF RATES OF EQUILIBRATION OF DEUTERIUM IN URINE AND SERUM IN SUBJECTS WITH ABNORMAL WATER DISTRIBUTION

SUBJECT	DIAGNOSIS	METHOD OF D_2O ADMINISTRATION	TIME FOR EQUILIBRATION† (HR.)
J. R. D.	Cardiac with decompensation	I.V.	11
H. C. M.	Cardiac with decompensation	P.O.	6
J. R.	Cardiac with decompensation	P.O.	4
C. F. K.	Cardiac with decompensation	I.V.	6
J. I. P.	Nutritional edema secondary to metastatic carcinoma	I.V.	8
E. J. H.	Nutritional edema secondary to metastatic carcinoma	I.V.	4
E. R. E.	Cirrhosis of the liver with ascites	I.V.	2
H. R. E.*	Panhypopituitarism secondary to craniopharyngioma	P.O.	6
R. K. W.*	Cushing's syndrome	P.O.	5

*Not edematous.
†Ratio of urine serum D_2O equaled 1.00 ± 0.02 at these times.

Since the D_2O concentration is the same in all body fluid after equilibration has occurred, there is no reason to exclude from D_2O studies patients who have a fever or patients who are sweating minimally. Objection could validly be raised in cases where profuse sweating was occurring within two to three hours following the administration of heavy water (the period during which equilibration is taking place), in patients with severe diabetes insipidus who might require a continuous water intake during this period, or in patients undergoing marked diuresis.

The advantages of using urine rather than serum are particularly important in field studies on large groups of men. If orally administered D_2O is used, venipuncture is omitted entirely. The voided urine samples may be sealed immediately, no further treatment being necessary prior to analysis. Moreover, since there is no significant change in D_2O concentration between three- and five-hour samples, accurate timing of the samples is not necessary.

SUMMARY

1. An accurate method for the recovery of urinary D_2O is presented.

2. The total body waters of thirteen normal male subjects obtained from simultaneous blood serum and urinary D_2O determinations are identical.

3. There is no difference in the values obtained from urine samples (obtained between three and five hours).

4. The D_2O lost in the urine during the period of equilibration is negligible.

5. Equilibration times of D_2O among the various body fluids in edematous subjects are long, and in such individuals the measurement of total body water required many hours with minimum fluid intake, and many samples for analysis.

Heavy water (99.5 per cent D_2O) was obtained from the Stuart Oxygen Company, San Francisco, California.

Preparation of the heavy water as isotonic saline solution, pyrogen-free, was done by Abbott Laboratories, North Chicago, Illinois.

We wish to express our appreciation to the Quartermaster Corps Climatic Research Laboratory for its continued interest and support of the project, to the Surgeon General's Office of the United States Army for its cooperation in supplying normal subjects, and to the Veterans Administration Hospital, Hines, Illinois, for the use of its facilities. We are also indebted to Professors Smith Freeman and Malcolm Dole for their valuable suggestions and criticism.

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LOCALIZATION OF AN INSULIN-NEUTRALIZING FACTOR BY ZONE ELECTROPHORESIS IN A SERUM OF AN INSULIN-RESISTANT PATIENT

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A NUMBER of diabetic patients have been reported to require excessively large amounts of insulin for control of their diabetes. In some instances the sera of such insulin-resistant patients have been shown to possess insulin-neutralizing activity, which was attributed to an antibody to insulin.[1-3] Recently, Stavitsky and Arquilla[4] have demonstrated the presence of specific antibodies to insulin in two insulin-resistant diabetics.

The sera of these patients agglutinated tannic acid-treated sheep erythrocytes to which insulin had been adsorbed and also lysed red cells to which the hormone had been coupled with bis-diazotized benzidine.

In the present study the serum of a diabetic patient, showing marked insulin resistance, was fractionated by zone electrophoresis, using starch as a supporting medium with a view to isolating the insulin-neutralizing factor and localizing it in a particular serum fraction.

The case is fully reported elsewhere.[5]

METHODS

Blood was obtained with the patient in the fasting state during a period when she had not received insulin for 18 days. During this fasting period she ran high blood sugars up to 390 mg. per cent. The glycosuria amounted to 160 Gm. per day but there was no ketonuria. No attempt was made to control the diabetes in order to obtain serum for these studies. Prior to this period her diabetic state was controlled with 500 units of crystalline zinc insulin daily in divided doses. The serum was separated and stored at –10° C. for ten months before electrophoretic separation was carried out. Precipitin, passive transfer, and skin tests were done at the time when the serum was obtained.

To remove the glucose present in the serum possessing insulin-neutralizing capacity, and in the serum of the controls, the sera were dialyzed at pH 7.4 against a phosphate-saline buffer of ionic strength 0.155 (the contribution of the sodium chloride to the ionic strength being 0.135) before injection into mice for testing. Blood sugar determination on these sera showed they contained less than 9 mg. per cent of reducing substances by the Folin-Wu method.

The technique used to demonstrate the presence of an insulin-neutralizing factor in the patient's serum and in the electrophoretically separated fractions was that described previously by Lowell.[1] A mixture of 0.2 ml. of serum and 0.01 unit of crystalline zinc insulin in 0.5 ml. saline was prepared immediately prior to use. The animals had been fasted for 18 hours but had had free access to water. Blood sugars were determined by the Folin-Wu

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method on 0.1 ml. samples obtained from the tail vein using 0.26 mg. heparin as anticoagulant. After an initial blood sample had been obtained the mixture of serum and insulin in a total volume of 0.7 ml. was injected intraperitoneally. Subsequent blood samples were obtained at 30 and 60 minutes. The values obtained at the 30- and 60-minute period varied from 10 to 15 mg. either way. As a consequence it was decided to use only a 45-minute specimen. A similar procedure was adopted for testing insulin-neutralizing capacity of the fractions separated by starch electrophoresis. In each set of experiments control tests were performed using serum from a diabetic patient well controlled on less than 40 units crystalline zinc insulin per day. This serum was taken 16 hours after insulin administration. Additional tests with this control serum and with the serum from the insulin-resistant patient to which saline instead of insulin was added were carried out.

The serum was analyzed at 0.8° C. by moving boundary electrophoresis at pH 8.6 in Veronal buffer of ionic strength 0.1[6] in a Tiselius apparatus, Spinco Model H.* Because of the high optical sensitivity of this apparatus the serum was diluted with buffer to 1 per cent protein concentration and dialyzed against the buffer for 48 hours prior to electrophoresis. The relative concentrations of the various components were calculated from the schlieren patterns by the method of Tiselius and Kabat.[7] The total protein concentrations of the serum, determined refractometrically,[6] was found to be 7.98 Gm. per cent as compared with 7.08 Gm. per cent determined by salt fractionation.[8]

For fractionation of the serum into albumin and the various globulin fractions—alpha, beta, and gamma—zone electrophoresis using starch as a supporting medium was used. The method, with slight modifications, was that described previously by Kunkel and Slater.[9] A trough 65 cm. by 5 cm. by 13 mm. was made of a glass plate, and two one-half inch Plexiglas bars were clamped to its sides. Thick wax paper was used for lining the trough. Filter-paper pads were inserted at the ends of the trough and served also as bridges between the trough and buffer vessels. The potato starch† was thoroughly washed twice with distilled water, and excess moisture was removed on a Buchner funnel. Finally, buffer solution was used as a displacing agent, providing an additional wash and resulting in the saturation of the starch with buffer ions. Sufficient buffer was then added to the starch to make it into a slurry. This was poured into the trough, excess moisture was removed by drying with filter papers, and, finally, the upper surface of the block was planed off with a wide spatula. Two milliliters of the serum to be separated were injected linearly across the middle of the block, the width of the zone of application being 20 mm. Application of the serum across the middle of the block prevented its displacement by chromatography and resulted in a sharpening of the zone of application during the two-hour equilibration with buffer which usually preceded electrophoresis. At the end of this period the upper surface of the block was covered with a sheet of wax paper to minimize evaporation.

The two buffer vessels filled to the same level were connected by filter-paper wicks to the electrode vessels containing silver-silver chloride electrodes in 20 per cent potassium chloride solution. These electrodes were used in preference to the conventional platinum or carbon and steel electrodes since no change in pH was found to occur. The buffer used was Veronal at pH 8.6 and ionic strength 0.05. This ionic strength was used rather than the usual ionic strength of 0.1 since it gave as good a resolution and allowed the application of higher voltages without undesirable increase in amperage. In this study two identical starch blocks, placed on the same buffer vessels, were prepared to separate simultaneously 4 ml. of the same serum. The potential applied was 400 volts and the total current for the two starch blocks was 28 Ma.

The electrophoretic separation was carried out in the cold room at 2° ± 1° C. The length of the experiment was 22 hours and the albumin peak was separated from that of the gamma globulin by 30 cm. The migration of the albumin could be easily seen due to the color of the various pigments associated with it. At the end of the experiment the region

*Manufactured by Specialized Instruments Corporation, Belmont, Calif.

†Amend potato starch for chromatography was found to be satisfactorily pure for these experiments.

over which the serum fractions were distributed was determined by applying a filter-paper strip along the surface of the block. After removal it was stained with bromphenol blue. This "print" revealed distinct bands corresponding to the various protein components.

For elution of the serum fractions from the starch, each block was cut into segments 1 cm. wide. These were placed in sintered glass funnels and eluted by displacement with saline under suction. It was found that the contamination of the eluates with small amounts of starch interfered with the determination of protein concentration. To prevent this, all eluates were centrifuged at 2,500 r.p.m. for about 10 minutes and the clear supernatant was decanted. The total volume of the eluate of each segment was about 7 ml. An equal aliquot of 1 ml. from each tube was used for protein determination by the method of Lowry and associates[10] and a plot of relative protein concentration led to a clear delineation of the zones containing the electrophoretically separable serum fractions. The various protein fractions were reconstituted by pooling the eluates as indicated by the graph and concentrating them by dialysis against 25 to 35 per cent dextran* solutions in standard Visking tubing. To reduce contamination of a fraction with material from fractions adjacent to it, only the eluates corresponding to the peaks in the protein curve were used for concentration. For comparison of the activity of the various fractions with that of untreated whole serum, all fractions were concentrated to the same volume as that originally used for electrophoretic separation.

To determine whether the various chemicals used in the procedure might have any insulin-neutralizing capacity by themselves when injected into the test mice, one "control fraction," devoid of protein, was prepared by pooling eluates from the zones adjacent to the gamma-2 globulin fraction and concentrating them by the same procedure.

RESULTS

From the data of Table I it is readily seen that the beta and particularly the gamma globulins of the serum obtained from the insulin-resistant patient were elevated in comparison with the normal values for sera of women.

TABLE I. SERUM PROTEIN ANALYSIS—ELECTROPHORETIC DISTRIBUTION IN PER CENT

	ALBUMIN	ALPHA$_1$ GLOBULIN	ALPHA$_2$ GLOBULIN	BETA GLOBULIN	GAMMA GLOBULIN	RATIO (A/G)
Insulin-resistant patient	51.22	3.61	10.28	15.0	19.89	1.05
Normal women*	58.40	5.17	9.81	12.42	14.18	1.40
(age 18-25)	±2.31	±0.73	±0.98	±1.18	±1.76	
20 normal women†	← 63.2 →		9.1	13.6	14.1	

*Results obtained in this laboratory.
†Data of H. E. Levens[13] referred to in *Plasma* 1: 582, 1953.

The data of Table II show the high insulin-neutralizing capacity of the whole serum from the insulin-resistant patient as compared to that of the control serum.

TABLE II

	SERUM FROM INSULIN-RESISTANT PATIENT PLUS		SERUM FROM CONTROL SUBJECT PLUS	
	SALINE	INSULIN	SALINE	INSULIN
Number of animals	6	5	5	6
Average % change in blood sugar	−7.8	+3.1	−7.1	−52.6
Standard deviation	26.30	7.44	15.07	14.73

*Supplied through the courtesy of Commercial Solvents Corporation, Terre Haute, Ind. This lot contained dextran with an average molecular weight of 60,000.

The electrophoretic separation on the starch blocks and the manner in which the separated serum was divided into albumin and the various globulins is indicated in Fig. 1. The electrophoresis on the two starch blocks resulted in identical separation, as was shown by the two protein curves. The gamma globulin fraction was found to migrate toward the cathode due to electro-osmosis, as reported previously by Kunkel and Slater.[9]

The results of injecting aliquots of the original serum, of the control serum, and of the various serum fractions obtained by electrophoresis are presented in Table III. The albumin, and alpha and beta globulin fractions showed almost an equal decrease in blood sugar as the control serum, thus demonstrating that no insulin-neutralizing activity was associated with these fractions. The gamma-2 globulin fraction had maximal insulin-neutralizing activity comparable to that of the original serum. The "control fraction" and the gamma-1 globulin had a comparable effect by partially inhibiting the hypoglycemia produced by insulin, but their effect was smaller than that produced by the gamma-2 globulin fraction.

The results of precipitin, passive transfer, and skin tests were negative.

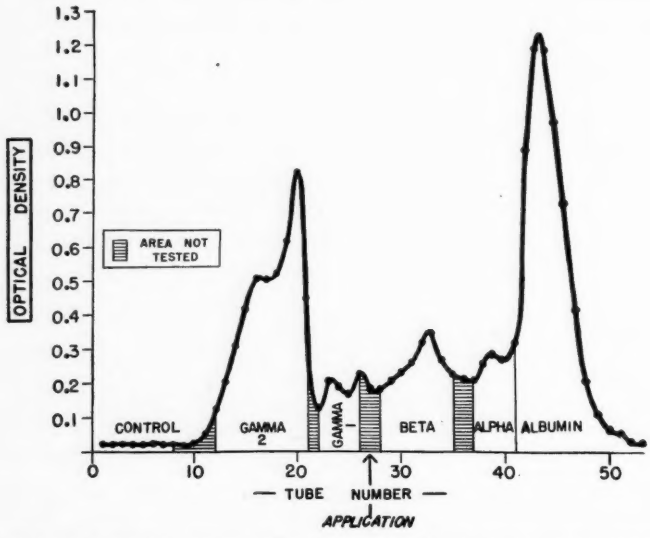

Fig. 1.—Protein distribution curve obtained by starch electrophoresis, showing the subdivision into various fractions.

DISCUSSION

The results of the free electrophoresis showed an elevation of gamma globulins in the serum of the insulin-resistant patient. Although immunization of various experimental animals with specific antigens has been shown to be associated with an elevation of the gamma globulins in the immune sera,[7] an increase in this globulin fraction cannot by itself be considered evidence for the presence of antibodies. In the present case no antibody to insulin could be demonstrated by the conventional methods, tests for both the precipitin and the skin-sensitizing antibody being negative. However, the electrophoretic separation of the

TABLE III

MATERIAL TESTED	NO. OF ANIMALS	AVERAGE % FALL OF BLOOD SUGAR	STANDARD DEVIATION	P VALUES		
				CONTROL FRACTION	$GAMMA_2$	$GAMMA_1$
Control fraction	14	22.6	16.15	—	—	—
Gamma-2	12	5.5	15.75	<0.05	—	<0.01
Gamma-1	8	27.5	13.27	—	<0.01	—
Beta	8	49.8	9.83	—	—	<0.01
Alpha	7	44.6	7.62	—	—	<0.01
Albumin	6	42.4	15.23	<0.05	<0.01	<0.01
Whole serum	7	6.4	10.39	—	—	—
Control serum	4	48.6	6.42	—	—	—

serum has enabled us to show that the insulin-neutralizing activity was mainly associated with the gamma-2 globulin fraction and that it was of the same order of magnitude as that found in the original serum. Zone electrophoresis has the additional advantages over the conventional methods of serum fractionation by allowing simultaneous separation of the serum into its electrophoretically separable components and the direct characterization of the various components. Furthermore, it is rapid and gentle. In the experiments presented here the various fractions recovered from the starch block may be considered to serve as internal controls, and the results of Table III indicate that the maximal insulin-neutralizing activity was in the gamma globulin fraction. It was completely absent in the albumin and the alpha and beta globulin fractions. The small insulin-neutralizing activity found in the two fractions adjacent to the gamma-2 globulin fraction, that is, the gamma-1 globulin fraction and the control fraction, may be interpreted as being due to small amounts of material from the gamma-2 globulin fraction being contained in the two fractions. Because of the unavailability of serum, further separation could not be carried out to clarify this point. The unequivocally high insulin-neutralizing capacity associated with the gamma-2 globulin fraction is in full accord with the findings of Fillipis and Iannacone[11] who showed that a gamma globulin fraction isolated by salt fractionation from an insulin-resistant serum contained the insulin-neutralizing factor. Although their gamma globulin fraction was stated to be electrophoretically 96 per cent pure, these workers did not state the nature of the contaminating material. We believe that in the present study starch electrophoresis has enabled us to localize this activity more directly and more specifically in the gamma-2 globulin fraction. The failure to demonstrate any allergic manifestations to insulin are in accord with the previously expressed view[3] that insulin-neutralizing factor might be due to an antibody distinct from that found in insulin allergy. Indeed, the recent work of Loveless and Cann,[12] who fractionated serum from an insulin-allergic patient by the electrophoresis-convection method, suggests that reaginic activity was almost exclusively associated with beta globulins.

Our results, in conjunction with the findings of other workers, support the belief that the insulin-neutralizing capacity in this serum from an insulin-resistant patient is of the nature of antibody to insulin and is associated with gamma globulins.

SUMMARY

The serum of an insulin-resistant patient was fractionated into various electrophoretically separable components by zone electrophoresis. Insulin-neutralizing activity was shown to be associated with the gamma globulin fraction.

We should like to acknowledge the skillful assistance of Mr. P. L. Rojowski in the electrophoretic separation and of Miss Eleanor Davidson for the help with the animal experiments.

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INHIBITION OF THE URICOSURIC ACTION OF BENEMID BY SALICYLATE

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RECENT studies of renal urate excretion in man indicate that plasma urate is completely filtered by the glomeruli, but that 90 to 95 per cent of the filtered urate is reabsorbed by the proximal tubules by active enzymatic processes.[1, 2] There are a number of drugs which inhibit this reabsorptive process and thereby increase the rate of excretion of urate. Of these so-called uricosuric agents the most effective and most innocuous is probenecid (Benemid), which is p-(di-n-propylsulfamyl)-benzoic acid. This drug has proved to be of significant value in the treatment of gout.[3-5]

In acute experiments in gouty subjects single oral doses of 2 Gm. of Benemid have been found by Sirota and his colleagues[2] to produce on an average a fourfold increase in the urate clearance within four hours. In more prolonged experiments by us with doses of 0.5 Gm. Benemid every six hours, the urate clearances became two to four times as great as the control values.[5] Under these circumstances the serum urate concentration fell 30 to 60 per cent within 24 to 72 hours.

Another effective uricosuric agent is salicylate, administered either in the form of sodium salicylate or acetylsalicylic acid. In doses of 3 to 7 Gm. daily, salicylate drugs increase the excretion of urate and significantly lower the serum urate levels in gouty as well as normal subjects. It might be expected that salicylates would act synergistically with Benemid to produce a greater uricosuric effect than with either drug alone. Surprisingly, the opposite effect was seen; the lowering of serum urate levels by Benemid was completely suppressed when salicylates were simultaneously administered. Mention of this phenomenon has already been made in our report on the use of Benemid in gout.[5] It was independently announced by Gutman and Yü[6] in a preliminary report.

In the present paper the experiments have been extended to determine whether the failure of the serum urate to fall was actually due to blocking of the uricosuric effect of Benemid or to falsely high urate levels in the presence of salicylate metabolic products. In addition an effort was made to determine the dosage and serum level of salicylate at which the blocking phenomenon occurs.

Since the completion of the experimental work for this paper, further corroboration of the suppressive effect of salicylate on the uricosuric action of Benemid has been offered in a report in abstract form by Sirota and Yü[7] on the basis of studies of renal clearance in acute experiments on gouty subjects.

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METHODS

Four normal subjects—i.e., nongouty convalescing hospital patients without evidence of cardiac or renal disturbance—were each given 4 Gm. of aspirin daily, in divided doses, for 4 days. Serum urate and salicylate analyses were made in the mornings before, during, and after salicylate administration. In four other normal subjects, aspirin was given for 4 days as above, and then 1 Gm. of Benemid twice a day was added to the aspirin regimen.

Four gouty subjects, maintained on a low-purine diet, were given 1 Gm. Benemid every 12 hours for 4 days. The drug was withdrawn after a good uricosuric effect was observed. When the serum urate level had returned to the control range, Benemid therapy was resumed, but in addition 5 Gm. of aspirin was administered daily. This regimen was continued for four to six days. Then the aspirin was withdrawn but the Benemid was continued. Analyses for serum urate were made each morning during the complete study, and for serum salicylate during the period of administration of aspirin. In two of these subjects collections of the 24-hour urine were made which were analyzed for urate both directly and after extraction of the acidified urine to remove gentisic acid as in the method used by Yü and Gutman.[8]

Two grams of Benemid was administered daily to one nongouty and three gouty subjects, until the lowered level of serum urate was stabilized. Then in addition to the Benemid, aspirin in successively raised daily doses of 1, 2, 3, and 4 Gm. was administered. Usually each dosage was maintained for three days. Serum urate and salicylate were determined each morning during the experiment.

Urate in serum and urine was analyzed by a photoelectric modification of the Folin method[9] and salicylate by the method of Brodie and associates.[10]

RESULTS

Table I summarizes the results in the eight normal subjects given aspirin first. They show that salicylate did not produce false elevations of the serum urate concentration, even though the serum salicylate rose as high as 28.0 mg. per 100 ml. Indeed, the serum urate levels fell significantly in each subject, as might be expected from the known uricosuric action of salicylate. Preliminary extraction of the Folin-Wu filtrates of these serum specimens did not effect any significant lowering of the urate concentration. Therefore, it was unlikely that gentisic acid or any other extractable derivative of salicylate that might act as a chromogen in urate analyses was present in significant concentrations. This finding is in conformity with the insignificantly low serum gentisic acid concentrations seen by Roseman and Dorfman[11] after administration of salicylate.

In the urine specimens obtained during salicylate medication, some metabolite of salicylic acid, presumably gentisic acid, was found in concentrations high enough to interfere with urate analyses. When the acidified specimens were extracted with ether and then analyzed for urate, the values were on the average 25 per cent (range 20.4 to 28.8 per cent) lower than those from the unextracted urine. These findings corroborate those of Gutman and Yü.[6] Urate analyses on urine specimens from subjects not on salicylate medication showed no essential differences before or after ether extraction.

Complete blocking of uricosuric action was seen in all instances in which Benemid and salicylate were given simultaneously. This response was recog-

TABLE I. CHANGES IN SERUM URATE IN NORMAL SUBJECTS GIVEN ASPIRIN ALONE OR ASPIRIN FIRST THEN ASPIRIN PLUS BENEMID

EXPERIMENT	TYPE OF SUBJECT	SERUM URATE (MG. PER 100 ML.)					MAXIMAL SERUM SALICYLATE (MG. PER 100 ML.)
		CONTROL	ASPIRIN ALONE	ASPIRIN PLUS BENEMID	BENEMID ALONE	NO DRUG	
1	Normal	5.6	3.4				25.5
2	Normal	3.9	3.0				28
3	Normal	5.0	4.2				28
4	Normal	3.0	1.3				17.5
5	Normal	3.2	1.5	3.0		3.8	23
6	Normal	4.2	2.8	4.5		3.6	29
7	Normal	3.1	1.7	3.2	1.8		22
8	Normal	3.9	2.5	3.4	2.5		45

nizable whether salicylates or Benemid was given first. The phenomenon occurred in both normal and gouty subjects. In Experiments 5 through 8 of Table I, the serum urate concentrations were reduced during four days of aspirin medication to an average of 59 per cent of their control values. Yet, when Benemid was added, the urate returned to and remained approximately at the control levels. When salicylate medication was stopped in two of these patients (Experiments 7 and 8), the urate concentration promptly decreased on Benemid to the low level seen on salicylate alone.

Table II summarizes the serum urate changes in the seven gouty and one normal subjects to whom was administered first Benemid then Benemid and aspirin. The pattern was the same in all instances. There was a fall in the serum urate concentration within 24 to 72 hours. The low level was maintained until Benemid was stopped (Experiments 9 through 12). Then the urate quickly rose to the initial hyperuricemic level. Now when 4 Gm. of aspirin was given along with 2 Gm. of Benemid daily, there was no significant change in the urate concentration. However, when salicylate medication was withdrawn, but that of Benemid maintained, the serum urate concentration was seen to drop as precipitously as before and to the same low range. In Experiments 13 through 16, similar blockage of uricosuric action was obtained with gradual additions of aspirin to the Benemid when the plasma salicylate became sufficiently high (Table II).

TABLE II. EFFECT OF ASPIRIN ON THE SERUM-LOWERING EFFECT OF BENEMID IN SEVEN GOUTY AND ONE NORMAL SUBJECTS

EXPERIMENT	TYPE OF SUBJECT	SERUM URATE (MG. PER 100 ML.)					MAXIMAL SERUM SALICYLATE (MG. PER 100 ML.)
		CONTROL	BENEMID ALONE	NO DRUG	BENEMID PLUS ASPIRIN	BENEMID ALONE	
9	Gouty	10.0	6.0	10.2	10.4	5.8	25
10	Gouty	10.2	4.4	8.4	10.6	4.1	22
11	Gouty	8.1	4.4	9.0	10.1	5.1	35
12	Gouty	14.4	7.4	15.0	16.3	6.0	35
13	Gouty	12.4	7.8		11.2		15.0
14	Gouty	7.9	4.1		7.4	3.9	11.5
15	Gouty	7.6	5.2		9.4	5.5	13.5
16	Normal	4.4	3.2		5.1	3.0	15.0

Relation of Urate Excretion to Serum Urate Concentration.—That the failure of serum urate to fall when aspirin and Benemid were given simultaneously was due to a blocking of the uricosuric effect rather than to any change in urate metabolism is demonstrated in the urate excretion studies in two of the gouty subjects. These are shown graphically in Fig. 1. The urinary urate values are those obtained by analyses of acidified 24-hour specimens after they had been extracted with ether to remove gentisic acid. It can be seen that the fall in serum urate concentration during Benemid medication alone was associated with a virtually doubled urate excretion, whereas the fixation of the high serum urate levels during combined Benemid and aspirin therapy was associated with the same low urate excretion as during the control periods.

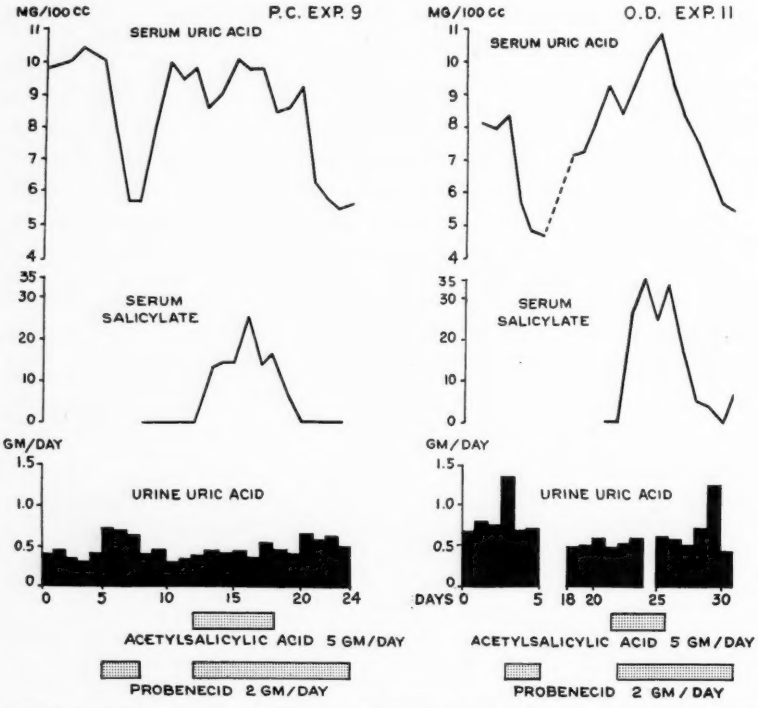

Fig. 1.—Urate excretion studies in relation to serum urate levels in two gouty subjects during probenecid and/or acetylsalicylic acid therapy.

Relation of Plasma Salicylate Level to Inhibition of Benemid Uricosuric Effect.—In Experiments 13 through 16, the dosage of aspirin was gradually increased to determine, if possible, the minimal serum salicylate level at which inhibition of uricosuria occurred. Three of the subjects were gouty; the fourth (Experiment 16) was a patient with nongouty osteoarthritis with normal urate levels. The results are shown in Figs. 2 and 3. It could be seen that salicylate levels below 5 mg. per 100 ml. did not influence the Benemid effect. In three of the four experiments the unequivocal rise in serum urate concentration occurred at salicylate levels around 10 mg. per 100 ml. In the fourth (Experiment 15) the rise was seen at 5 mg. per 100 ml. It must be recognized, how-

ever, that the salicylate concentration was determined on the morning following the day's medication, and it does not, therefore, represent the maximal level achieved for the particular dosage. It is probably safe to conclude from these limited data that sustained serum salicylate concentrations between 5 and 10 mg. per 100 ml. are adequate to produce a complete annulment of the uricosuric action of Benemid. These findings appear to be in accord with the

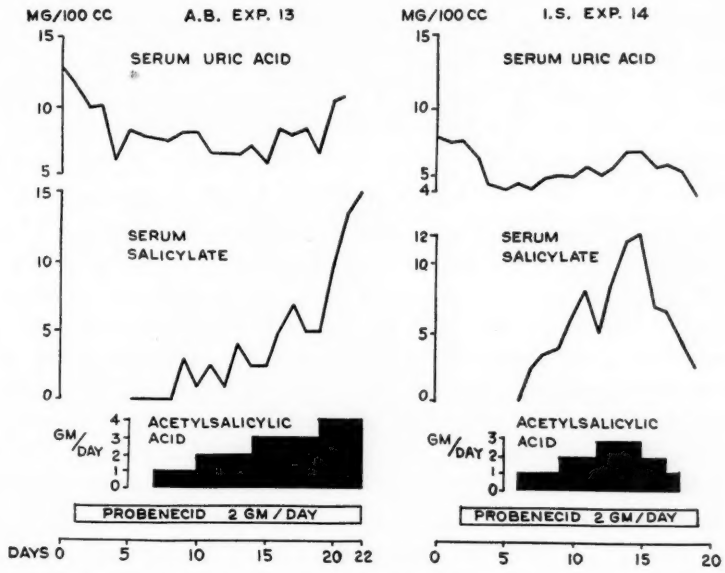

Fig. 2.—Relation of serum salicylate level to inhibition of uricosuric action of probenecid in two gouty subjects.

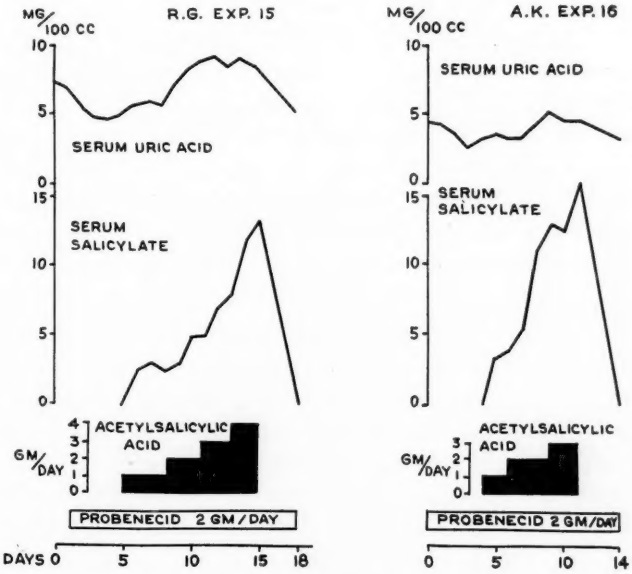

Fig. 3.—Relation of serum salicylate level to inhibition of uricosuric action of probenecid in one gouty and one nongouty subject.

report of Sirota and Yü[7] that the maximal depression by salicylate in acute experiments occurred at serum salicylate levels below 15 mg. per 100 ml. We found no upper limit for the depressive effect; it was still maximally present at serum salicylate levels of 45 mg. per 100 ml.

COMMENT

It is difficult to visualize how the two substances, salicylate and Benemid, each of which inhibits tubular reabsorption of urate, can act to annul the inhibitory effect of each other. Modern studies of biologic antagonism lead to the belief that the uricosuric effect of Benemid is due to its competition with some substrate in one of the enzymatic reactions involved directly or indirectly in tubular transfer of urate.[12] Probably salicylate acts in a similar manner, but upon a different substrate, for it seems a priori unlikely that Benemid and salicylate could compete with each other for the same competitive effect on a third substance and each succeed in completely annulling the other's competitive effect.

Whether the facts that the bulk of excreted salicylate is in the form of salicyl glycuronide or salicyluric acid and that these conjugates are probably produced in the renal tubules and secreted by them[13] have any bearing on the inhibitory effect of salicylate is a matter of conjecture. Benemid reaches the tubules by tubular reabsorption.[12] It is conceivable that salicylate interferes with some type of conjugation of Benemid which might be prerequisite to its uricosuric effect. Yet this would not explain the effect of Benemid in inhibiting the uricosuric action of salicylate. Just as difficult to explain is the finding that salicylate does not interfere with the action of Benemid in raising the blood levels of penicillin by preventing the tubular excretion of this antibiotic.[16]

It has been suggested that salicylate owes its therapeutic action to stimulation of production of adrenocortical hormones, since both eosinopenia and increased urate excretion occur four to six hours after a large dose of salicylate.[14] But the serum salicylate level in these experiments was 30 mg. per 100 ml. or higher. Since, in the author's experience,[14a] uricosuria can be produced with much lower levels of salicylate (10 to 20 mg. per 100 ml.), and can, at least in some patients, be maintained for months or even years without any evidence of hyperadrenalism, it is more likely that the usual uricosuric effect of salicylate is due to direct action of the drug on the renal tubules rather than to the effect of adrenocortical hormone on these structures. At any rate the problem of explaining the mutual antagonism of salicylate and Benemid would not be easier if salicylate acted only indirectly through adrenocortical stimulation.

From a clinical point of view the finding that the inhibitory effect of salicylate does not occur until sustained plasma salicylate concentrations between 5 and 10 mg. per 100 ml. are reached minimizes the danger of the sporadic use of aspirin for pain or headache during Benemid therapy. The usual dose of 0.67 Gm. (10 grains) produces a maximal plasma salicylate concentration of 2 to 4 mg. per 100 ml., which within 1 or 2 hours falls to insignificant levels.[15] On the other hand, since even such small doses of salicylate, if given

at frequent intervals for a number of days, will produce a pyramiding of the plasma salicylate to high levels, prolonged salicylate is contraindicated in Benemid therapy.

SUMMARY

In both gouty and nongouty subjects, administration of salicylate during Benemid therapy annuls the lowering of serum urate induced by Benemid. Similarly, Benemid annuls the lowering of serum urate levels caused by salicylate medication. This mutual inhibition is shown by urine excretion studies to be due to interference with uricosuric action. Apparently salicylate competes with Benemid for some antagonistic effect on tubular enzymatic processes involved in urate reabsorption.

The inhibitory effect of salicylate begins to occur at sustained plasma salicylate levels between 5 and 10 mg. per 100 ml.

During salicylate medication, plasma urate analyses are not affected by the presence of salicylate derivatives, but urine urate values are about 25 per cent too high, unless gentisic acid is first removed by extraction with ether.

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A COMPARISON OF THE NEURATH AND THE TREPONEMAL IMMOBILIZATION TESTS AND THEIR CORRELATION WITH ROUTINE SEROLOGIC TESTS

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THE problem of the biologic false positive (BFP) serologic reaction has been recognized since tests for the diagnosis of syphilis were first developed. The importance of this problem has become more generally recognized since the widespread adoption of serologic testing for premarital, prenatal, pre-employment and preinduction examinations.[1-5] Moore and Mohr[4] reported that 45 per cent of 300 patients with positive serologic reactions, and no other evidence of syphilis, gave negative reactions with the treponemal immobilization (TPI) test. Nelson[5] reported that 42 per cent of 529 untreated Navy personnel, with positive Kahn tests, gave negative reactions with the TPI test. The problem of BFP reactions with routine serologic tests makes necessary the availability of a specific confirmatory test.

Many serologic tests have been developed to differentiate the syphilitic individual from one giving a BFP reaction. All these tests, including the Kahn verification techniques, have proved unsatisfactory.[2, 6, 7] The Neurath test,[8] described in 1947, and the TPI test,[9] described in 1948, now offer promise of solving this difficult problem.

Neurath[8] reported that when 285 nonsyphilitic patients giving positive routine serologic tests were tested by his method, 92 per cent gave BFP reactions, 6 per cent gave inconclusive results, and 2 per cent demonstrated a syphilitic response. The positive control group of 327 confirmed syphilitic patients yielded the following reactions: 95 per cent syphilitic, 2 per cent BFP, and 3 per cent inconclusive.

General agreement currently exists in the literature regarding the specificity of the TPI test for the treponemal infections of syphilis, yaws, pinta, and bejel.[10-14] The availability of the TPI test is, nevertheless, sharply limited by its complexity. Currently only 7 serologic laboratories in the United States perform the test. The Neurath method, in contrast, is adaptable to any laboratory currently performing routine serologic examinations. If the results of the Neurath and the TPI tests are comparable, the former would be a valuable aid in solving the problem of the BFP reactor. For this reason, the authors decided to compare the technically adaptable Neurath test with the relatively complex TPI test.

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METHODS

1. *Patients.*—One hundred and forty-four patients were studied, 85 of whom were classified as having syphilis. All the diagnoses of latent syphilis and of syphilis unqualified were based on the positive results of the TPI test. These diagnoses were considered valid since our experience with the sera of known syphilitic and nonsyphilitic patients has verified the accuracy of the TPI test. The syphilitic patients, except one who had received treatment, reacted positively with one or more of the routine serologic tests for syphilis. The syphilitic diagnoses are indicated in Table I.

TABLE I. DIAGNOSES OF PATIENTS WITH SYPHILIS

DIAGNOSES	NUMBER OF PATIENTS
Primary syphilis	0
Secondary syphilis	1
Congenital syphilis	1
Tabes dorsalis	3
Aortic aneurysm	1
Other forms of cardiovascular syphilis	3
Central nervous system syphilis, asymptomatic	12
Syphilis, unqualified*	18
Latent syphilis	46
Total	85

*These patients did not have spinal fluid examinations, or physical examinations were incomplete.

Of the 61 patients with negative TPI tests, 39 had positive routine serologic tests. Eight of the latter group had received treatment for syphilis in the past. Fourteen additional patients, negative by routine serologic tests, presented various physical findings suggestive of syphilis. Two of these patients had received treatment for syphilis, and another had received penicillin therapy for an unrelated condition. Six patients had physical examinations which were not indicative of syphilis, and their routine serologic tests were negative.

2. *The Neurath Euglobulin-Inhibition Test.*—This is a quantitative flocculation test for syphilis utilizing the euglobulin fraction of serum. The euglobulin is first precipitated from the whole serum and then redissolved in a phosphate buffer as described by Neurath and associates.[15] The phosphate buffer solution of globulin is then diluted, utilizing buffered saline as the diluent in one series and the "inhibitor," described below, as the diluent in a second series. The dilutions were then used to perform the standard flocculation tests for syphilis. Identical results in the two series are indicative of a syphilitic type of reaction. Weaker reactions in the "inhibitor" series are classified as BFP reactions. The substances causing BFP reactions, having a greater affinity for the inhibitor, are not available to react with the antigen. Syphilitic reagin, having no affinity for the inhibitor, produces identical reactions in both series.

The "inhibitor" is an alpha globulin fraction with the properties of serum lecithin.*

Each globulin dilution was tested with the Mazzini lipoidal antigen and the VDRL and Rein-Bossak cardiolipin antigens. The methods used were those described by the authors.[16-18] In addition, a routine quantitative flocculation test utilizing the VDRL antigen was performed on the whole serum. The reactions are classified in Table II.

3. *The Treponema Pallidum Immobilization Test.*—This, the most specific test for syphilis yet developed,[10, 12, 13, 14] was performed by Dr. Ruth Boak and James N. Miller.** The technique employed was that recommended by Nelson and Mayer[19] and by Magnuson and Thompson[20] except that a new medium[21] for extraction of spirochetes was used; i.e., normal rabbit serum inactivated at 60° C. for 45 minutes and diluted 50 per cent with 0.85

*Provided through the courtesy of Dr. Neurath.

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per cent physiologic salt solution. In addition, the test as outlined below was performed in duplicate. The patient's serum was placed in each of two tubes and a suspension of living *Treponema pallidum* was added to each tube. Fresh complement was added to the "test" tube and inactivated complement to the "control" tube. The tubes were incubated for 16 hours under an atmospheric tension of 5 per cent CO_2 and 95 per cent N_2. Specimens from each tube were then examined by dark-field microscope and the percentage of motile spirochetes in each tube was determined. The percentage of spirochetes immobilized by the syphilis antibody was determined by means of the following formula: Percentage of spirochetes immobilized by syphilis antibody equals.

$$\frac{\text{Percentage motile in "control" tube less percentage motile in "test" tube}}{\text{Percentage motile in "control" tube}}$$

Method of reporting:

Positive—50 per cent or more of the spirochetes immobilized.
Doubtful—21 to 49 per cent of the spirochetes immobilized.
Negative—0 to 20 per cent of the spirochetes immobilized.

Inconclusive or invalid—most of the spirochetes in the "control" tube are immobilized by antibiotics or some toxic substances in the serum.

4. *Serologic Tests for Syphilis.*—All sera were tested with Kahn and Kolmer lipoidal antigens and both Kline and Kolmer cardiolipin antigens.

TABLE II. TYPES OF NEURATH REACTIONS*

TYPE OF REACTION	ANTIGEN PLUS SERUM	ANTIGEN PLUS EUGLOBULIN IN BUFFER	ANTIGEN PLUS EUGLOBULIN IN INHIBITOR
Syphilitic			
a. Syphilitic	44430	44430	4443±
b. Weakly syphilitic	20000	20000	20000
c. Syphilitic type with substance giving BFP reaction	44432	42000	42000
Nonsyphilitic			
d. Negative	00000	00000	00000
e. BFP type	44200	44200	00000
f. Negative type with substance giving BFP reaction	42100	00000	00000
Inconclusive			
g. Inconclusive	10000	±0000	32000
h. Inconclusive with substance giving BFP reaction	44432	00000	42000

*Reactions c, f, and h were not described by Neurath; however, they are presented in this paper as samples of types of reactions frequently observed in this study.

RESULTS AND DISCUSSION

The purpose of this paper is to compare the agreement of the TPI and Neurath tests and their correlation with routine tests for syphilis. We have not attempted to evaluate the accuracy of the TPI and Neurath tests, since the latter objective was precluded by the character of this series of patients. Seventy-nine per cent of the patients were 45 years of age or older, and many of them were unable to recall their histories with any convincing degree of reliability. A vague history of a penile chancre could not be accepted as indisputable evidence for a diagnosis of syphilis.

1. *Neurath-TPI Comparison.*—The Neurath and TPI tests agreed in 88 patients (61.1 per cent) and disagreed in 20 patients (13.9 per cent). The

remaining 36 patients (25 per cent) gave inconclusive results with the Neurath test. If this latter group be eliminated from the comparison, as the results in this group by the two tests cannot be compared, agreement is found with 81.5 per cent and disagreement with 18.5 per cent of the remaining 108 cases. The results are summarized in Table III. Roy and associates[22] in a similar study reported agreement in 67.7 per cent and disagreement in 11.5 per cent of 96 cases. A summary of the treatment, physical findings, and serologic reports in relation to the Neurath and TPI tests is recorded in Table IV. Fifteen (21.4 per cent) of the 70 patients with syphilitic types of Neurath tests (Table III) had negative TPI tests. Seven of this group of 15 patients had received treatment for syphilis (Table IV). Five of them had received treatment because of unconfirmed histories of penile lesions while the remaining 2 patients had received treatment because of persistently positive routine serologic tests for syphilis. One patient in this group of 15, who had not received treatment, had positive routine serologic reports and a perforated nasal septum attributable to inhalation of chromic acid fumes. The 3 patients with positive TPI reports and negative Neurath tests had central nervous system syphilis. The 2 patients with doubtful TPI reports and negative Neurath tests were completely asymptomatic.

TABLE III. COMPARISON OF NEURATH AND TPI TESTS

TPI	TOTAL PATIENTS	NEURATH		
		SYPHILITIC	NONSYPHILITIC	INCONCLUSIVE
Positive	72	52	3	17
Doubtful	11	3	2	6
Negative	61	15	33	13
Total	144	70	38	36

Agreement: 88. TPI positive or doubtful with Neurath of the syphilitic type; and TPI negative, Neurath negative.

Disagreement: 30. TPI positive and doubtful with Neurath negative; and TPI negative with syphilitic type of Neurath reaction.

Inconclusive Neurath reports: 36.

Thirty-five patients showed a much stronger reaction with the whole serum than with the globulin fraction of the Neurath test. Nineteen of them had a syphilitic type of Neurath reaction. Thirteen of the 19 patients had positive TPI tests and it was concluded that syphilitic antibody as well as a substance yielding BFP reactions was present. A reaction was found with the serum but not the globulin fraction with 16 patients. These were considered to be nonsyphilitic reactions, since no reagin was precipitated in the globulin fraction. Seven of these 16 patients, however, gave positive TPI tests.

2. *Consistency of Neurath and TPI Reports.*—Additional specimens were taken from 21 patients for repeat Neurath tests and from 25 patients for repeat TPI tests. Repeat specimens were taken, when possible, from patients whenever the TPI and Neurath reports disagreed, when one report was invalid or inconclusive and when the TPI reports were doubtful. When both the Neurath and TPI tests agreed, repeat examinations were not performed because of the limited laboratory facilities for performance of the latter test. From Table V it will be

TABLE IV. SEROLOGIC REACTIONS AS RELATED TO TREATMENT AND PHYSICAL EXAMINATIONS

TPI AND NEURATH TESTS	NUMBER OF PATIENTS	TREATED			NOT TREATED				
		NUMBER OF PATIENTS	TREATED FOR SYPHILIS	TREATED WITH PENICILLIN FOR OTHER CONDITIONS	NUMBER OF PATIENTS	PHYSICAL EXAMINATION SUGGESTIVE OF SYPHILIS*		PHYSICAL EXAMINATION NEGATIVE FOR SYPHILIS	
						SEROLOGY POSITIVE	SEROLOGY NEGATIVE	SEROLOGY POSITIVE	SEROLOGY NEGATIVE
Agreement									
TPI Positive Neurath Positive	52	32	27	5	20	5	0	15	0
TPI Doubtful Neurath Positive	3	1	1	0	2	0	0	2	0
TPI Negative Neurath Negative	33	4	4	0	29	5	5	13	6
Total	88	37	32	5	51	10	5	30	6
Disagreement									
TPI Positive Neurath Negative	3	2	2	0	1	1	0	0	0
TPI Doubtful Neurath Negative	2	0	0	0	2	0	0	2	0
TPI Negative Neurath Positive	15	9	7	2	6	1	1	4	0
Total	20	11	9	2	9	2	1	6	0
Neurath Inconclusive									
TPI Positive	17	12	9	3	5	5	0	0	0
TPI Doubtful	6	4	4	0	2	2	0	0	0
TPI Negative	13	2	2	0	11	4	0	3	4
Total	36	18	15	3	18	11	0	3	4
Total	144	66	56	10	78	23	6	39	10

*Physical or neurologic findings sometimes associated with, but not specific for syphilis.

TABLE V. REPRODUCIBILITY OF RESULTS

RESULTS	NEURATH TEST	TPI TEST
Confirmed	13 (81.3%)	18 (85.7%)
TPI doubtful, then positive	0	2 (9.5%)
Conflicting results with same test	3 (18.7%)	1 (4.8%)
Total	16	21
One specimen invalid or inconclusive	5	4

TABLE VI. SEROLOGIC REACTIONS* AND THE TPI TEST

TPI TESTS	NUMBER OF PATIENTS	KOLMER			KOLMER CARDIOLIPIN			KAHN		
		POS.	VARIABLE†	NEG.	POS.	VARIABLE†	NEG.	POS.	VARIABLE†	NEG.
Positive	72	39 (54%)	11 (15%)	22 (31%)	61 (85%)	8 (11%)	3 (4%)	28 (32%)	21 (29%)	23 (39%)
Doubtful	11	3 (27%)	3 (27%)	5 (46%)	7 (46%)	0	4 (36%)	3 (27%)	3 (27%)	5 (46%)
Negative	61	10 (17%)	11 (18%)	40 (65%)	20 (33%)	9 (15%)	32 (52%)	13 (21%)	16 (26%)	32 (53%)
Total	144	52	25	67	88	17	39	44	40	60

*Results of the Kline cardiolipin antigen test were not tabulated. Positive results were found with all specimens showing positive serologic tests with one or more of the other antigens.

†These reports refer to patients whose sera, over a period of years, have shown both positive and negative reactions.

noted that specimens from 5 patients were inconclusive with the Neurath test and specimens from 4 patients were invalid with the TPI test. The table compares the reproducibility of results for the Neurath test in 16 patients and for the TPI test in 21 patients.

3. *Relation of Serologic Reactions to the TPI Tests.*—The relationship between reactions with antigens routinely used in serologic tests for syphilis and those of the TPI test are indicated in Table VI. All sera which showed positive results with one or more of the tabulated antigens also showed positive reactions with the Kline cardiolipin antigen. It is apparent that the Kolmer cardiolipin antigen is the most sensitive of the three antigens tabulated. This antigen gave consistently positive reactions with 85 per cent of the sera which showed positive reactions with the TPI test. There were only 4 per cent negative reactions with sera giving positive TPI tests. The Kahn and Kolmer antigens, on the other hand, gave 31 per cent and 39 per cent negative reactions, respectively, with the same sera.

The increased sensitivity of the Kolmer cardiolipin antigen is achieved with the sacrifice of its specificity. This is indicated by the fact that, when specimens giving negative TPI reports were tested with the Kolmer cardiolipin antigen, many more consistently positive reactions were observed than were seen with the other two antigens.

Serum tests based solely upon the results of testing with Kahn and Kolmer antigens, as is commonly practiced in many laboratories, would have missed a significant number of untreated TPI positive or doubtful patients. The sera of 7 untreated patients, who gave positive TPI examinations and that of one who gave a doubtful reaction with that test, were negative when tested with the Kahn and Kolmer antigens. All were positive when tested with the Kolmer car-

diolipin antigen. Had the latter antigen not been used, these cases might very well have been given a clean bill of health with potentially disastrous consequences to them.

Thirty-eight (62 per cent) of the patients who had negative TPI tests showed reactions with one or more of the antigens used in the serologic tests for syphilis. These figures are not surprising since, in the majority of these patients, the positive routine serum test was the sole factor suggestive of syphilis.

CONCLUSION

The Neurath and TPI tests were compared in 144 patients. They agreed in 61.1 per cent and disagreed in 13.9 per cent of the entire group. Results of the Neurath test were inconclusive with the remaining 25 per cent of the patients. The Neurath test, as performed in this laboratory, was of no practical value in differentiating BFP from syphilitic reactions.

Serum tests based solely upon the results of testing with Kahn and Kolmer antigens would have missed a significant number of untreated TPI positive or doubtful patients.

Results were more readily reproducible with the TPI test than with the Neurath test.

Cardiolipin antigens were more sensitive than the lipoidal Kahn and Kolmer antigens. However, they were less specific, so positive results must be interpreted with caution.

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EFFECT OF HEPARIN ON THE DISTRIBUTION OF INTRAVENOUSLY ADMINISTERED C^{14}-LABELED SOYBEAN OIL EMULSION IN RATS*

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INTRODUCTION

IN 1943 Hahn[1] discovered that injection of heparin into animals displaying alimentary lipemia caused rapid disappearance of the turbidity of the blood. Subsequent investigations by other workers[2-5] revealed that heparin injection caused transformation of turbid lipoprotein to nonturbid substances, and this reaction has been intensively studied both in vitro and in vivo. It has also been shown[6-9] that the disappearance of lipemic turbidity following heparin injection is accompanied by a decrease in lipid concentration in the blood, indicating that injection of heparin not only transforms turbid lipoprotein into nonturbid forms but also accelerates the removal of lipid from the blood. The question of the fate of the fat removed from the blood under the influence of heparin has received little attention.

Previous work in this laboratory[6] demonstrated the rapid removal from the blood of intravenously infused fat emulsion after the administration of heparin in rats. The rapidity and magnitude of this removal were such that to explain it on the basis of rapid oxidation seemed unlikely. Furthermore, gravimetric analysis of liver and spleen indicated that, under the influence of heparin, the concentration of lipid in these organs actually decreased. Assay of other fat tissues with the gravimetric method proved to be inadequately sensitive to detect changes in composition following heparin. The use of C^{14} as a tracer substance, however, provides a more precise means of determining the sites of fixation of fatty substances. The value of this method as a means of studying the metabolic pathways of infused fat emulsions has been demonstrated previously by Lerner and his group[10] and Geyer and co-workers.[11] Both these groups utilized singly labeled C^{14} synthetic triglycerides as contrasted to the homogeneously labeled C^{14}-soybean oil employed in the present study.

EXPERIMENTAL METHODS AND PROCEDURES

I. *Preparation of Emulsion.*—C^{14}-labeled soybean oil, obtained by biosynthesis as previously described,[12] was used as the tracer material in these experiments. The oil contained 69.9 μc of C^{14} per gram of carbon and 54.3 μc per gram of oil when analyzed by

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combustion and gas-counting techniques.[12] By dilution, combustion, and counting under the same conditions used to assay experimental samples in the present study, the specific activity of the oil was 3.56×10^4 counts per minute per infinitely thick sample.

The oil was injected as a 2.5 per cent emulsion in 5 per cent glucose with 1.0 per cent to 1.2 per cent Tween 60 as emulsifying agent. Twenty to twenty-one milliliters of 5 per cent glucose and 0.20 to 0.25 ml. of Tween 60 were weighed in a tared, covered homogenizer cup. Six-tenths milliliter of the C^{14}-soybean oil was added and the final weight taken. Composition of the emulsion was expressed as weight of oil divided by the total weight of the emulsion times 100. Emulsification was achieved by mixing in a Sorvall Omnimixer at 14,000 rpm for 3 to 5 five-minute periods. The emulsions were examined under a dark-field microscope before use and contained no particles larger than 3 microns in diameter, with the vast majority of particles being less than 1 micron in diameter. Each batch of emulsion was enough for 2 experimental animals and was used within 2 hours after preparation.

II. *Injection of Animals.*—Two male, Sprague-Dawley albino rats, weighing 200 to 235 grams, but weighing within 15 grams of each other, were deprived of food for 3 to 4 hours before use. Each animal was injected with 4 ml. of emulsion per 100 grams of body weight, which is equivalent to 1 Gm. of oil per kilogram of body weight. The actual amount of emulsion received was determined by weighing the syringe before and after the injection. One of each pair of rats was injected with 10 mg. of heparin per kilogram of body weight 15 minutes prior to the injection of emulsion. The heparin solution was prepared immediately before use by dissolving 1 mg. of powdered heparin sodium salt (obtained from Connaught Laboratories) per milliliter of isotonic saline solution. All injections were made into the lateral tail vein without anesthesia while the animal was restrained in a specially designed cage.

III. *Collection of Respiratory CO_2 and Preparation of Tissue Samples.*—Immediately after injection of the C^{14}-fat emulsion, each animal was placed in a metabolic chamber with a volume of 2.5 liters, through which CO_2-free air was passed at a rate of 500 to 600 ml. per minute. The respiratory CO_2 was trapped in 10 per cent aqueous NaOH in a tower with a sintered glass scrubbing disk. The animal remained in the chamber for 30 minutes, after which time it was removed and anesthetized with ether. The abdominal aorta was cannulated in both directions with polyethylene tubing. Blood was collected from the upper cannula, and through the lower cannula the animal was perfused first with 30 ml. of a suspension of washed blood cells in 6 per cent dextran solution (20 per cent hematocrit) containing glucose and electrolytes in the proportions found in Locke's solution, and then with 30 ml. of Locke's solution in 6 per cent dextran. Perfusion of the whole animal was deemed necessary because of the large proportion of injected radioactivity remaining in the blood of the animals which did not receive heparin. If this procedure were not carried out, radioactivity trapped in the tissues in residual blood could be confused with radioactivity fixed by the tissue. After the completion of perfusion, the animal was dispatched by opening the chest cavity. The total elapsed time from the injection of the emulsion to the death of the animal averaged 50 minutes.

The liver, lung, and spleen were removed, and samples of the small intestine, skin, subcutaneous fat, retroperitoneal fat, and gastrocnemius muscle were taken. Three to four milliliters of blood had previously been collected in oxalate-containing tubes from the cannula nearest the heart immediately before the perfusion began.

The tissues were blotted and weighed on a Roller-Smith torsion balance and were analyzed for total lipid content by a modification of the Majonnier technique.[13] Three milliliters of whole blood was placed in 75 ml. of Bloor's alchol-ether mixture. The mixture was heated on the steam bath for 1 hour, and filtered through Whatman No. 1 paper. The residue and paper were thoroughly washed with diethyl ether. The combined extracts were evaporated to dryness under a stream of N_2 and the residue extracted with warm diethyl

ether. This extract was filtered into a tared vessel, with thorough washing of the filter paper, and again evaporated under a stream of N_2. The residue was dried in vacuo and weighed, with the result expressed as milligrams of total lipid per milliliter of whole blood.

IV. *Combustion, Plating, and Counting of C^{14}-Fat Samples.*—Four- to 6-milligram portions of the dry residue derived from the Majonnier lipid analysis of the tissue samples and from the lipid analyses of whole blood were weighed into platinum boats. The fat samples were thoroughly mixed by stirring with a stainless steel spatula before being transferred to the boats. The samples were burned in a microcombustion apparatus under a stream of CO_2-free O_2 and with the aid of a platinum catalyst. The resultant CO_2 was trapped in 6 ml. of a solution made by diluting 2.0 ml. of saturated NaOH and 10.0 ml. of 1 molar $BaCl_2$-2 per cent NH_4Cl solution to 100 ml. with CO_2-free water. The $BaCO_3$ was then filtered with gentle suction on tared sintered stainless-steel disks (Micro-Metallic Corp., porosity G2) held in a stainless-steel filtering tube, dried in vacuo, and weighed. Each milligram of lipid yielded 10 to 11 mg. of $BaCO_3$, which is near the theoretical yield of 12 mg. $BaCO_3$ per milligram based on a carbon content of 75 per cent. NH_4Cl was included in the absorbing solution to ensure a perfectly flat surface of the $BaCO_3$ sample when plated under these conditions, and the combination of sintered steel disks and NH_4Cl virtually eliminated the troublesome phenomenon of "charge build-up" sometimes encountered during counting of solid $BaCO_3$ samples. With the geometry inherent in our counting conditions, 40 mg. samples of $BaCO_3$ proved to be "infinitely thick."

The respiratory $C^{14}O_2$ was assayed by precipitating $BaCO_3$ from a suitable aliquot of the solution used to trap CO_2 from the respiratory chamber. It was often difficult to prepare $BaCO_3$ plates of the respiratory CO_2 which did not crack or flake due to the large excess of NaOH in the suspension, but the addition of extra NH_4Cl, combined with minimum washing of the precipitate during filtration, considerably reduced the number of failures. The total amount of respiratory CO_2 excreted was determined gravimetrically by quantitative precipitation of $BaCO_3$ in large aliquots of the solution and collection of the precipitate on tared sintered glass crucibles. The $BaC^{14}O_3$ samples were then counted in a Tracerlab windowless gas-flow counter, using 99 per cent helium—1 per cent isobutane as the counting gas, connected to a Tracerlab Superscaler. The samples were counted long enough to reduce the statistical error to 1 per cent. All counts were corrected for coincidence loss, infinite thickness, and background.

V. *Calculations.*—In order to compare the radioactivity appearing in the lipid isolated from tissue samples with that contained in the injected emulsion, the "total" activity of a fat sample was arbitrarily defined as specific activity, expressed as counts per minute per infinitely thick $BaCO_3$ precipitate, multiplied by the weight of the fat sample. Thus the per cent of the injected dose appearing in a fat sample could be calculated by dividing the quantity [cpm × mg. fat] by the quantity [mg. emulsion injected × mg. oil per mg. emulsion × 3.56×10^4 cpm] and multiplying by 100.

To compare the radioactivity appearing in the respiratory CO_2 with that contained in the injected emulsion, the following equation was used:

$$\frac{\text{mg. carbon in } CO_2 \text{ collected} \times \text{cmp}}{\text{mg. oil injected} \times \text{mg. carbon per mg. oil} \times 3.56 \times 10^4 \text{ cpm}} \times 100 = \text{per cent injected radioactivity in respiratory } CO_2$$

RESULTS

Table I shows the per cent of the injected radioactivity recovered from the liver, lung, spleen, and blood of three pairs of animals. As might be expected from our previous results obtained by chemical determination of fat,[6] the amount of C^{14} lipid recovered from the whole blood and the spleen of the heparinized animals was much less than that recovered from the nonheparinized animals. The calculation of C^{14} recovery in whole blood was based on the assumption[14] that 8 per cent of the body weight is circulating whole blood. This assumption

was probably in error, as the summation of the per cent C^{14} recovered, including the respiratory $C^{14}O_2$, from nonheparinized animals always exceeded 100 per cent. C^{14}-lipid recovery in the liver was slightly increased in the heparinized animals, but at most accounted for a relatively small portion of the radioactivity removed from the blood and spleen. C^{14}-lipid fixation in the lung appeared to be unaltered by heparin.

TABLE I. EFFECT OF HEPARIN ON PER CENT C^{14} ACTIVITY* RECOVERED FROM TOTAL ORGANS AND BLOOD AFTER INJECTION OF C^{14}-SOYBEAN OIL EMULSION

	EXPERIMENTS 1 AND 2		EXPERIMENTS 3 AND 4		EXPERIMENTS 5 AND 6	
	HEPARIN	CONTROL	HEPARIN	CONTROL	HEPARIN	CONTROL
Liver	23.9	21.8	36.4	17.1	22.5	16.1
Spleen	0.86	4.2	1.70	3.58	0.45	2.06
Lung	1.16	1.53	1.24	0.85	0.49	0.54
Blood	15.8	83.0	8.82	92.3	36.7	91.0

*Per cent activity recovered from organ $= \frac{\text{cpm} \times \text{mg. fat in organ}}{\text{cpm} \times \text{mg. oil injected}} \times 100$

Table II shows the per cent of the injected radioactivity recovered from the lipid isolated from skeletal muscle, small intestine, and skin. All three of these tissues, but especially muscle, showed a rise in C^{14}-lipid content in the heparinized animals. The specific activity of the lipid isolated from subcutaneous and retroperitoneal fat depots showed a small rise with heparin, but these tissues cannot be directly compared with the three listed in Table II because of the great variability in fat content of such depot tissues. On the other hand, muscle and skin account for a large portion of body weight and represent tissues of low- and high-fat content, respectively. It is felt that C^{14}-lipid fixation in these tissues is indicative of generalized C^{14}-lipid fixation.

TABLE II. EFFECT OF HEPARIN ON PERCENTAGE C^{14} ACTIVITY* RECOVERED PER GRAM OF TISSUE AFTER INJECTION OF C^{14} SOYBEAN OIL EMULSION

	EXPERIMENTS 1 AND 2		EXPERIMENTS 3 AND 4		EXPERIMENTS 5 AND 6	
	HEPARIN	CONTROL	HEPARIN	CONTROL	HEPARIN	CONTROL
Muscle	0.112	0.017	0.134	0.034	0.159	0.042
Intestine	0.192	0.133	0.230	0.102	0.311	0.149
Skin	0.137	0.066	0.181	0.139	0.173	0.059

*Percentage C^{14} activity recovered per gram of tissue $= \frac{\text{mg. fat per Gm. tissue} \times \text{cpm}}{\text{mg. oil injected} \times \text{cpm}} \times 100$

TABLE III. EFFECT OF HEPARIN ON PERCENTAGE C^{14} RECOVERED FROM RESPIRATORY CO_2 AFTER INJECTION OF C^{14}-SOYBEAN OIL EMULSION

PAIR NO.	CONTROL	HEPARINIZED
1	0.70	4.66
2	1.28	2.51
3	1.91	1.84
4	1.16	4.71
5	0.95	3.56

Periods of collection were 30 minutes in each instance.

Table III shows the C^{14} recovered from the respiratory CO_2 of five pairs of animals during the first 30 minutes after injection of emulsion. In all but

one instance, the C^{14} appearing as CO_2 was markedly increased in the heparinized animals. Although the increased C^{14} in CO_2 accounts for only a small portion of the C^{14} removed from the blood under the influence of heparin, it does indicate that in the heparinized animals more C^{14} lipid was presented to tissues to be available for oxidation.

DISCUSSION

The results of this study confirm our previous observations[6] that under the influence of heparin, fat administered as an emulsion is rapidly removed from the blood.

The data from these experiments cannot be compared directly with those of previously reported C^{14} tracer work in this field[10, 11] because of the homogeneously labeled material used and the shorter periods of observation. The brief interval in these experiments was selected on the basis of preliminary studies which showed that the maximal difference in blood C^{14}-lipid levels between the control and heparinized animals occurred within the first hour. The enhancing effect of heparin on rate of uptake of fat varied among the tissues studied. The decreased uptake by the spleen can probably be attributed to transformation of the fat to a nonparticulate form, thus reducing trapping and phagocytic engulfment. The relatively greater effect of heparin on fat uptake by muscle than on uptake by other tissues studied suggests that the action of heparin is selective. If, as has been proposed,[15-18] the heparin mechanism participates in normal fat metabolism, the administration of exogenous heparin might be expected to increase the rate of uptake by all tissues without altering relative amounts. However, the present findings do not necessarily speak against a physiologic role for heparin in fat metabolism because tissues may vary considerably in their ability to respond to a physiologic agent.

The marked rise in C^{14} in the respiratory CO_2 indicates that heparin speeds the oxidation of injected fat emulsion. Whether this is associated with a general rise in fat oxidation or is a selective action on injected fat emulsion cannot be decided from the present studies. A study of the effect of heparin on total oxygen consumption and respiratory quotient should answer this question. Since postheparin plasma is lipolytic,[19-21] and since tissues probably oxidize free fatty acid rather than triglyceride, the augmentation of oxidation of injected fat emulsion by heparin may be due to increased rate of presentation of fatty acid to tissues.

SUMMARY AND CONCLUSIONS

C^{14}-labeled soybean oil emulsion was given intravenously to 10 rats, 5 of which had previously been heparinized. The experiments were carried out in pairs so that in each experiment the same batch of freshly prepared emulsion was used for both the control and the heparinized animal. In 2 pairs, only respiratory CO_2 was studied. In the other 3 pairs, following a 30-minute period of CO_2 collection, viviperfusion was carried out, and then tissue samples of organs and fat tissues were taken for analysis of C^{14} fat fixation. Comparison of heparinized and nonheparinized animals revealed that heparin produced the

following changes in C^{14} fixation: a profound decrease in blood and spleen, a marked rise in respiratory CO_2 and muscle, a moderate rise in intestine, small and variable rises in liver and skin, and small and inconsistent changes in lung. The results indicate that heparin accelerates the movement of fat from blood to tissues and thereby makes the fat more rapidly available to oxidative processes.

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INFLUENCE OF CHELATES ON THE METABOLISM OF RADIOYTTRIUM (Y-90)

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INTRODUCTION

RECENT years have seen the development of the chemistry of the chelates, more specifically, the amino poly carboxylic acids (Martell and Calvin,[1] Chaberek and Bersworth[2]). This class of compounds offers to the investigator a group of new tools for the study of mineral metabolism in a variety of fields (Weinstein, Robbins, and Perkins[19]). This is occasioned by the ability of chelates to form soluble, dialyzable compounds which do not yield appreciable amounts of metal ions and, therefore, do not react as do the more common compounds (i.e., Cl, NO_3, CO_3, etc.). The metabolic fate of these chelated materials may differ from the usual for the unchelated metal, and it was postulated that they might differ according to the chelating agent, their function being influenced predominantly by the ionization constant, and in turn by the degree with which the metal being studied could be replaced by other ions, particularly serum sodium and calcum.

Several groups of investigators have studied the metabolic fate of yttrium, using Y-90 or Y-91, both carrier-free and with various amounts of the nonradioactive element (Y-89) added. These studies were made with the chloride, hydroxide, citrate, nitrilo triacetic acid, and ethylenediamine tetraacetic acid complexes. In general these findings have shown that when the total mass of yttrium administered was small, the radioactive material often concentrated in the skeleton (Bloom,[3] Hamilton,[4] Dobson,[5] Schubert,[6] Kelly,[7] Kidman,[8] Walker,[9] Rayner,[10] Cohn,[11] Lewin,[12] Greenberg,[14] Schubert[20]). As the total yttrium was increased, the chloride, hydroxide, and citrate often remained in situ on intraperitoneal or intrapleural injection, or were deposited in the liver and spleen if given intravenously. Since Y-90 offers possibilities of medical application, due to its pure energetic beta emission, it has been suggested as a means of reducing ascitic and pleural effusion in carcinomatosis (Lewin and associates[13]).

In view of these findings and our interest in bone metabolism, we began a study to determine whether readily obtainable pile-produced Y-90 (with

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carrier)* could be chelated and caused to concentrate selectively in proliferating bone. This paper reports on the metabolism of yttrium in rabbits and includes (a) screening of 6 complexing materials, (b) determination of influence of time on tissue concentration of yttrium, and (c) influence of amount of chelate on yttrium deposition in various tissues.

METHODS AND MATERIALS

Animal Studies.—Preliminary studies with rats were carried out by injecting subcutaneously into the right hind leg 1 ml. of YCl_3 (Y-90) solution chelated with one of the following materials: citrate; ethylenediamine tetraacetic acid (EDTA); N'hydroxy ethylenediamine triacetic acid (ED-ol); N, N'dihydroxy ethylenediamine diacetic acid (ED-diol); or N, N'dihydroxyethylglycine (EG-diol). Eight animals for each compound tested were sacrificed and the relative concentration of Y-90 determined by beta counting of selected oven-dried tissue.

A second screening procedure (Table I) was carried out by injecting intravenously into the marginal ear vein of rabbits, each of the previously listed Y-90 chelate solutions, at pH 6.0 – 6.5. Two animals for each compound tested were sacrificed and the relative concentration of Y-90 determined by beta counting representative oven-dried samples of the liver, kidney, spleen, cortical bone, and marrow. On completion of this series, one group of 4 rabbits were injected intravenously with Y-90 chelated with nitrilo triacetic acid (NTA) and the relative Y-90 content of the same tissues was determined.

Results of the first rat and rabbit series were used as a guide, and a study was made of the three chelates which had produced the highest bone/marrow and bone/liver ratios, i.e., EDTA, ED-ol, ED-diol. In this group of rabbits the amount of chelate material was increased in order to determine the effect of an excess of the complexing agent. The amount of chelate was *four times* the amount necessary to prevent precipitation of $Y(OH)_3$, at pH 6.5 – 7.0. More soft tissues were taken, as well as representative bone samples from the ribs, skull, and long bones (cortex and trabeculae of femur and tibia). The relative concentration of Y-90 was determined by beta counting, and is reported in Table II, as net counts per minute per gram of oven-dried tissue. The per cent of original dose remaining in liver at time of death and that excreted in urine and feces (48 hours) were determined. Four rabbits were used for each of the compounds tested.

In the third series of rabbits, the chelate (ED-ol) which previously had produced the greatest degree of deposition of yttrium in bone was used to

*Pile-produced:
Natural occurring Y-89—100%
Y-89 (n, γ) Y-90 (Barns 1.4)
Y-90
β- 2.18 mev. T½-61 hours
Zr-90
Available from Brookhaven National Laboratories as Y_2O_3, specific activity 0.65 mc. Y-90/mg.Y.
As daughter of Sr-90 (Fission Product)
Sr-90
β- 0.54 mev. T½-20 years
Y-90 (carrier-free)
Available with added carrier in limited amounts, Abbott Laboratories.

complex the Y-90 solutions. In order to determine the effect of lowered chelate concentration, *only sufficient* ED-ol was used to prevent precipitation of the $Y(OH)_3$, as the solution was brought to pH 6.5. To determine the effect of time on Y concentration, animals were sacrificed on the second, seventh, and fourteenth days after intravenous injection of the chelated Y-90 into the marginal ear vein. A total of 12 rabbits were injected and 4 killed at each of the 3 time intervals. The concentration of yttrium was determined by beta counting oven-dried samples, comparing with standard samples, and the Y content estimated as micrograms Y per gram dried tissue.

Preparation of Y-90 Solutions.—Finely divided spectroscopically pure Y_2O_3 was irradiated in the Brookhaven reactor for 5 to 7 days, at a thermal neutron flux of approximately 3.4×10^{12} n/cm.^{2}/sec. The specific activity obtained was 0.65 - 0.68 mc. Y-90/mg. Y.

The activated oxide was dissolved in 20 per cent HCl, made to volume with distilled water, and suitable aliquot pipetted into a 25 ml. volumetric flask. Solutions of each of the chelating agents were prepared, and a quantity of each was added to one of the volumetric flasks together with a few drops of brom-cresol purple indicator. The resultant acid solutions were adjusted to pH 6.0 - 6.5 with NaOH and the volume brought to 25 ml. One milliliter of these solutions was used for parenteral administration to rats and rabbits, and caused no general toxic reaction, localized irritation, or slough.

The amount of chelate was varied from just that amount necessary to prevent precipitation of $Y(OH)_3$ at pH 7.0, to four times this quantity. Solutions were obtained in all cases in which no precipitate formed on prolonged standing.

When Y_2O_3 is dissolved in HCl, a stable solution is obtained at pH 1-3. However, in a solution containing no chelating agent, $Y(OH)_3$ begins to precipitate as the pH increases so that at pH 6.0–6.5, essentially all the yttrium is present as the insoluble hydroxide. This helps to explain why injection of the chloride causes the yttrium to remain in situ. In the present studies, since the YCl_3 solutions are brought to pH 6.0–6.5, essentially what was injected was suspension of $Y(OH)_3$, which dialyzed slowly, and which in the case of rats remained at the site of subcutaneous injection.

The beta decay of solutions of Y-90 which were administered, as well as samples of bone and stomach from treated rabbits, have been followed for as long as 8 half lives ($T\frac{1}{2}$-61 hours) and no significant amount of long-life contaminants has been found. These findings indicate that the initial Y_2O_3 was of high purity and that the beta counts give a true indication of the yttrium content of tissues.

Standard samples of each of the solutions were prepared by diluting at a ratio of 2,000:1. These solutions were added to solution blanks and tissue blanks to obtain quantitative results as shown later in this article.

In Vitro Dialysis Studies.—Solutions of the yttrium compounds mentioned previously were prepared by adding to a YCl_3 solution *four times* the amount of complexing agent necessary to prevent precipitation of $Y(OH)_3$ when the solutions were brought to pH 6.0 – 6.5 with NaOH. Five milliliters of these solutions was mixed with 1 ml. of 5 per cent human serum albumin, pipetted into cellophane tubing, the tubing tied, and the whole dialyzed against 200 ml. of distilled water for 72 hours. The dialysis flasks were shaken periodically to ensure complete mixing. An aliquot of the water solution was analyzed each day and indicated continued dialysis to 72 hours. At the end of this time the per cent of Y which had passed the cellophane membrane was determined by beta counting the Y-90.

The design of this experiment was to determine whether yttrium chelated at pH 6 to 7 would be bound by a protein, such that the yttrium would not

dialyze through a semipermeable membrane. The technique described herein did not give a measure of the rate at which yttrium equilibrates with the dialysate, nor whether the protein effected the final equilibrium. However, the results do indicate that dialysis readily takes place under the conditions of both excess protein and chelate.

Analytic and Counting Procedures.—To determine the relative Y-90 content of tissues, two thinly sliced 2 to 5 gram representative samples of each fresh tissue were taken from each animal, placed in weighed planchets, dried for 12 hours at 120° C., again weighed, and the beta emission determined by counting with an end-window Geiger tube (3 mg./cm.^{2} mica, background 25-30 cpm, tube to sample 10 mm.). The observed counts were corrected for decay (T½-61 hours), to some common time, usually the time of death. The counts so obtained yielded the net counts per minute per gram of dried tissue (error ± 5 per cent). These values are an index of the Y-90 content and are semiquantitative in nature, since no correction was made for self-absorption by the samples.

To obtain a quantitative estimation of the yttrium content of tissues, aliquots of the solutions which were injected were mixed with lean beef, and 2 to 5 grams of these tissue samples were weighed, counted, and the net counts per microgram of added yttrium determined. Under these conditions the net counts obtained from Y-90 were a linear function of the added yttrium (Y-89 + Y-90) in the counting range (1,000 - 4,000 cpm).

To obtain an accurate estimate of the total quantity of Y-90 in the liver, the entire liver was removed and weighed, and a 20 gram aliquot of fresh tissue was dissolved in 100 ml. of concentrated HNO_3. This solution was diluted to 200 ml. and a 2 ml. aliquot pipetted into an aluminum planchet, dried, and the beta emission determined. The basis for quantitative estimation was a sample of lean beef to which had been added a known quantity of the Y-90 solution used to inject the animals, and carried through an identical procedure.

The excretion of Y-90 by rabbit was determined by collecting all of the urine and feces together for 48 hours. This was removed, the cage screen scraped, the pan rinsed with water, and the entire mass mixed with a Waring blendor. The weight of the mixture was determined, and two 50 gram aliquots dissolved in concentrated HNO_3, diluted to 200 ml., and 2 ml. dried and counted as indicated for the liver. The quantitative estimation of excretion (as per cent of original dose) was determined by comparison with "knowns" prepared by adding a quantity of the injection solutions to samples of nonradioactive feces, and carrying through the same procedure as outlined for the "unknowns."

RESULTS

In Vitro Dialysis Studies.—Dialysis of Y-90 solutions, in the presence of protein, shows that various chelates form complexes of sufficient stability and molecular size to permit them to pass a cellophane membrane. The results indicate that YCl_3 at pH 6.5 – 7.0 is not readily soluble (27 per cent), whereas 84 per cent of the citrate and 88 to 100 per cent of the chelates dialyzed within 72 hours. A value of 100 per cent indicates complete equilibrium.

Rat Studies.—Subcutaneous injection of YCl_3 and the yttrium complexes listed previously has shown that the chloride is largely retained at the site of injection, the citrate is markedly concentrated in the liver, spleen, and kidneys, while the chelates show varying degrees of affinity for bone. Details of these findings are omitted, as the results with rabbits confirm and extend the studies with rats.

Rabbit Studies.—In the results of the rabbit studies shown in Table I, it will be noted that intravenous injection of the citrate or ED-diol produced high

TABLE I. RELATIVE CONCENTRATION OF Y-90 IN TISSUES OF RABBITS. INFLUENCE OF CHELATING COMPOUNDS

COMPOUND*	LIVER	KIDNEY	SPLEEN	MARROW	CORTICAL BONE	CORT. BONE/ MARROW RATIO
			CTS./MIN./GRAM DRIED TISSUE† (× 1,000)			
Citrate	84.10	9.2	34.10	19.40	4.1	0.21
EDTA	0.95	4.2	1.30	0.20	5.8	29.00
ED-ol	0.98	3.3	1.40	0.46	7.9	17.10
ED-diol	45.00	22.7	30.80	11.90	3.7	0.31
EG-diol	0.61	6.0	0.86	0.40	6.0	15.00

*All materials at pH 6.0—6.5 administered intravenously into marginal ear vein of rabbits which were killed 48 hours after injection. Each solution contained *twice* the amount of chelate necessary to prevent precipitation of $Y(OH)_3$ at pH 6.5—7.0.

†Values shown are observed net counts (beta) per minute per gram of oven-dried tissue. All values shown are the mean of 4 or more determinations carried out on tissue taken from 2 rabbits for each compound tested. Dose: 100 microcuries Y-90, 0.30 mg. Y/kg.

concentrations of Y-90 in the liver, spleen, and bone marrow, 48 hours after administration. On the other hand, EDTA, ED-ol, and EG-diol concentrated relatively little in the liver, spleen, and bone marrow and produced high cortical bone/marrow ratios (15-29). In this series *double* the necessary amount of chelate was used to ensure complete solution. Details of the findings with NTA are not included, as they were completed at a later time. They, however, essentially duplicated those of ED-diol, giving relatively high concentration in liver, spleen, and marrow.

In the second series of rabbits (Table II), killed 48 hours after injection with EDTA, ED-ol, and EG-diol, there was a marked degree of uptake

TABLE II. RELATIVE CONCENTRATION OF Y-90 IN RABBIT TISSUES. INFLUENCE OF CHELATING COMPOUNDS

	EDTA*	ED-OL	EG-DIOL
	NET CTS./MIN./GRAM OVEN-DRIED TISSUE (× 1,000)		
Soft Tissue			
Glut. muscle	0.13	0.18	0.13
Heart muscle	0.61	0.71	0.37
Brain	0.38	0.22	0.29
Liver	1.50	4.30	1.40
Spleen	2.20	2.80	21.00
Kidney	10.70	7.00	13.00
Bone†			
Marrow	4.70	3.80	1.40
Rib	31.00	173.70	51.70
Cranium	23.00	103.00	31.70
Cortex	17.10	56.30	32.00
Trabecular	45.30	154.90	43.40
Per cent dose remaining in liver after 48 hr.	2.30	4.50	2.00
Per cent dose excreted in feces and urine, 48 hr.	27.80	23.80	47.60

*All materials at pH 6.0—6.5 administered intravenously into marginal ear vein. Rabbits killed 48 hours after injection. Four animals used for each compound. Results shown are mean of 8 determinations, duplicate samples from each rabbit. Each solution contained *4 times* the amount of chelate necessary to prevent precipitation of $Y(OH)_3$. Dose: 100 microcuries Y-90, 0.30 mg. Y/kg.

†Bones cleaned of connective tissue. Marrow removed from femur, cortical and trabecular bone separated and washed free of red marrow.

of yttrium by the ribs, cranium, and trabecular bone of the femur, particularly in the rabbits treated with ED-ol. Trabecular bone/marrow ratios ranged from 9.6 for EDTA to 40.7 for ED-ol. In the ED-ol-treated rabbits 23.8 per cent of the injected yttrium was excreted during 48 hours, and only 4.5 per cent of the dose remained in the liver at the time of death. A *fourfold excess* of chelate was used to complex the Y-90 solutions in each case.

TABLE III. YTTRIUM CONCENTRATION OF RABBIT TISSUE. INFLUENCE OF TIME OF DEATH*

TISSUE	TIME AFTER INJECTION		
	2 DAYS	7 DAYS	14 DAYS
	MICROGRAMS Y PER GRAM OF OVEN-DRIED TISSUE		
Blood	0.07	0.05	0.09
Brain	0.04	0.24	0.16
Muscle	0.48	0.22	0.33
Lung	1.65	2.36	3.70
Heart	1.48	1.11	2.90
Fat (perirenal)	0.22	0.15	0.22
Stomach	43.60	30.60	48.10
Small intestine	1.59	1.48	1.14
Large intestine	0.43	0.14	0.70
Liver	4.60	3.00	2.30
Spleen	10.50	3.40	4.90
Kidney	6.80	4.30	3.10
Skin	2.80	2.60	2.30
Rib	5.20	6.70	9.90
Sternum	2.50	3.30	3.70
Cortical bone	2.20	3.90	5.40
Trabecular bone	7.20	5.70	14.30
Skull	3.70	4.60	8.40
Marrow	2.90	0.47	0.42

*Animals killed at indicated intervals. Results are mean of eight samples of tissues taken from 4 rabbits for each time, total 12 rabbits (3.0—3.5 kg.). Chelate material, ED-ol, *just sufficient* used to prevent precipitation of $Y(OH)_3$ at pH 6.0—6.5. Dose: 100 microcuries Y-90, 0.40 mg. Y/kg.

In the third series of rabbits (Table III) only the *minimum* quantity of ED-ol necessary to complex the yttrium was used. Following intravenous injection of this complex, it was found that under these conditions only moderate amounts of yttrium were deposited in the bone (trabecular bone/marrow ratio at 48 hours was only 2.5). The stomach (free of contents), however, exhibited a high concentration of Y. With time (2 to 14 days) stomach and bones increased their Y content, spleen, liver, and kidney decreased, while other soft tissues generally retained that found 48 hours after injection.

Examination of the results shown in Tables I and II indicates that of the complexing materials studied N'hydroxy ethylenediamine triacetic acid (ED-ol) promotes greatest bone deposition of yttrium. Examination of the results shown in Tables II and III indicates that an excess of chelate (ED-ol) is necessary to promote greatest bone/marrow ratios.

DISCUSSION

When ionic solutions are parenterally injected into a viable organism, there occurs immediately, and in a relatively small volume, a shift of the pH

to 7.4, otherwise local tissue destruction occurs which may be followed by death, or localized slough. In general, those ions which tend to coagulate proteins will form the proteinate, while those which precipitate near neutrality will form the oxide or hydroxide. These reactions tend to give a suspension of relatively insoluble, finely divided material, which will be handled by the organism as would any inert particulate matter; the initial distribution being similar to that of an inert suspension or colloid (i.e., carbon black); however, the solubility product [A] [OH] or [A] [Protein] will largely govern the subsequent metabolic fate. Therefore, the solutions of many metallic *ions* when given intravenously will be initially collected in the spleen, liver, and bone marrow.

There is ample pharmacologic evidence to indicate that the total mass of ions administered (as milligrams per kilogram) markedly affects the distribution and excretion pattern (Bruner and associates,[15] Dudley,[16] Bloom,[3] Lewin and co-workers[12, 13]). This is particularly true of the carrier-free radioisotopes which are not normally present, and which may be given in easily measured doses, but having only negligible weight. Therefore, the solubility of a metal under physiologic conditions is a major factor in determining its metabolic fate. This is readily demonstrated by the use of a minute mass of a radioisotope, wherein one obtains a definite distribution pattern, but with the addition of increasing amounts of the same but nonradioactive ion (i.e., carrier) a point is reached at which the body fluids are saturated with respect to the [Metal]. With the addition of further carrier a different biologic pattern may be produced due to localized supersaturation with respect to [Metal]. Our purpose in this investigation has been to overcome the tendency of yttrium compounds to form particulate aggregates at pH 7.4, complexing in such a manner that the Y may be metabolized, and still not undergo simple precipitation.

The basis for this study has been a desire to obtain a radioactive material which would concentrate in proliferating bone. The effectiveness of gallium (as Ga-72) in therapy trials, even as a palliative measure, has been limited both by the toxicity of the carrier Ga and the whole body irradiation due to the energetic gamma emission (2.0-2.5 mev.) (King and associates,[17] Brucer and co-workers[18]). In view of the favorable radiation characteristics of Y-90 as indicated previously (see footnote, p. 793) it was postulated that some chelate might be found which would promote selective deposition of yttrium in bone, yet having low adsorption ratios in the liver, spleen, and particularly the bone marrow. When the Y was complexed with an excess of ED-ol this situation appears to be satisfied.

The findings that yttrium can also be made to concentrate in stomach tissue are being explored further in this laboratory, and it seems probable that by control of chelate concentration it may be possible to influence the site of major deposits of yttrium in the stomach or bone, still sparing the marrow.

SUMMARY

1. A study of the influence of various chelating agents on the metabolism of yttrium (as Y-90; half life 61 hours; 2.2 mev. beta) has shown that by selection of the proper reagent one may promote preferential deposition in selected tissue within 48 hours.

2. Yttrium complexed with citrate, ED-diol, or NTA deposited in the liver, spleen, and bone marrow of rabbits. Complexed with an *excess* of EDTA, EG-diol, or ED-ol, the yttrium is preferentially deposited in bone, with ED-ol giving the highest bone/soft tissue ratios.

3. Yttrium complexed with a *minimum* quantity of ED-ol is markedly concentrated in the wall of the stomach.

4. Radio yttrium (Y-90), administered intravenously as the chelate, is suggested as a means of delivering ionizing radiation of high energy to selected tissues, particularly to bone and stomach.

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A COMPARISON OF ROUTINE PLASMA VOLUME DETERMINATION METHODS USING RADIOIODINATED HUMAN SERUM ALBUMIN* AND EVANS BLUE DYE (T-1824)

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INTRODUCTION

EVANS blue dye (T-1824) has been used extensively for blood and plasma volume determination for more than thirty years. Dawson, Evans, and Whipple[1] reported in 1920 that T-1824 (orthotolidine combined with 2 mol. 1.8 amido 2.4 disulfonic acid) was the most suitable of more than sixty blue-azo and red dyes tested for blood and plasma volume determinations. Keith and Rowntree[2] reported a vital red dye method in 1916 and are considered to be the originators of the dye method for blood and plasma volume determination. Gibson and Evans[3, 4] reported further studies on many dye substances as late as 1936 and concluded with Dawson and associates that T-1824 remained the most suitable dye for blood volume work. Gregerson[5] published a practical modification in 1944 of the T-1824 method for use by the United States Army. This modification has become the standard procedure for the determination of blood and plasma volume with Evans blue dye in most laboratories. The universal acceptance of this dye method has led to its use as a standard of comparison for newer plasma volume estimation methods, including the use of radioactive isotopes. Schultz and co-workers[6] have reported a critical comparison in human subjects, and Sear and associates[7] have done comparative studies with experimental animals (dogs), both using radioiodinated human serum albumin (RISA) and Evans blue dye. Gray[8] has used T-1824 in his pilot experiments with radioactive chromic chloride plasma volume determinations. Evans blue dye was used in this laboratory for control purposes in the establishment of a gravimetric modification of the RISA method for plasma volume determination.[9] The object of this report is to compare critically the relative degrees of accuracy inherent in the two methods (gravimetric RISA vs. Evans blue) when used as routine clinical laboratory procedures.

METHODS

Standard solutions of RISA were prepared following the method of Zipf, Webber, and Grove,[9] and the optical density of the Evans blue dye was standardized according to the modification of Gregerson.[5] A 20 μc dose of RISA and a 10 ml. dose of Evans blue dye solution were prepared in separate syringes for injection. A preinjection sample of 7 ml. of blood

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was drawn without stasis from an antecubital vein of the subject. The syringe was removed, leaving the needle in the vein, and the blood placed into a 10 ml. heparin tube. The solutions of RISA and Evans blue dye were then injected consecutively through the same needle, flushing out each syringe with venous blood to ensure complete delivery of the dye and isotope solutions. Blood samples of 10 ml. were drawn without stasis at fifteen and twenty minutes postinjection. Each sample was divided into two parts by placing 5 ml. in each of two heparin tubes. The heparin tubes were carefully marked to ensure identification of the fifteen- and twenty-minute samples. Radioactivity assay and plasma volume determinations were performed following the gravimetric method of Zipf, Webber, and Grove.[9] The heparin tubes containing the control, fifteen- and twenty-minute samples for spectrophotometric dye concentration analysis were submitted to the biochemistry section of the clinical laboratory of the Miami Valley Hospital. Dilutions of control and dye-tinged plasma were prepared and the optical densities were determined. Plasma volumes were calculated by research laboratory personnel from optical density data received from the biochemistry section. Statistical analysis of each set of plasma volume data derived from each method of volume estimation consisted of the calculation of the average plasma volume, the standard deviation, the probable error, and the per cent probable error. Subjects for these comparative tests included seventeen normal adult males, thirteen normal adult females, and three adult male patients from the medical and surgical departments of the Miami Valley Hospital. These latter three patients were being treated for polycythemia vera, severe anemia of undetermined etiology, and bleeding peptic ulcer, respectively.

RESULTS

The results of thirty-four simultaneous plasma volume determinations with RISA and T-1824 dye on thirty-three different subjects are tabulated in Table I.

Schultz[6] and Sear[7] in their comparative studies with RISA and Evans blue dye found that the dye gave slightly higher values than RISA, but these differences appeared to be statistically insignificant. Comparisons in these cases[6,7] were made on the basis of *single* dye and isotope determinations on each subject. Table I shows the differences found in the series of comparisons obtained at the Research Laboratory of the Miami Valley Hospital. These comparisons were made from *two independent* blood samples drawn at fifteen and twenty minutes postinjection. This method of comparison afforded two independent plasma volume values for Evans blue dye and four values (two direct and two indirect) for RISA. Gregerson[10] has reported that 5 to 8 per cent of T-1824 is excreted per hour; therefore, it is apparent that no significant loss of dye occurs in the five-minute interval between samples and that this method of sampling does, in fact, provide two independent samples for comparison. Plasma volumes determined with Evans blue dye were 25.5 per cent higher on the average than those obtained with RISA. Gregerson[5] implied that the average plasma volume per kilogram of body weight is about 45 ml. per kilogram, but our studies show an average plasma volume of 41.5 ml. per kilogram by the RISA method and 52.1 ml. per kilogram by the T-1824 method. Gibson[11] obtained an average of 47.7 ml. per kilogram from forty cases. Keith[2] found an average of 50.0 ml. per kilogram from fifty cases, while other investigators[4] have obtained values as low as 41.5 ml. per kilogram with the dye method. Storaasli[12] obtained an average of 40.3 ml. per kilogram in thirty-one cases, and Berson[13] found an average of 47.5 ml. per kilogram in fifty-three cases using RISA alone. Comparison of the probable errors of precision of the two methods shows a significant difference. The T-1824 method showed an average probable error

TABLE I

PATIENT	AGE	WT. (KG.)	RADIOIODINATED HUMAN SERUM ALBUMIN							EVANS BLUE DYE					DIFFERENCE EVANS BLUE AND RISA	
			INDIRECT		DIRECT											
			15 MIN.	20 MIN.	15 MIN.	20 MIN.	AVERAGE	ML. P. E.	% P. E.	15 MIN.	20 MIN.	AVERAGE	ML. P. E.	% P. E.	ML.	%
J. G.	40	77.9	2725	2460	2607	2564	2589	90.6	3.50	4440	5620	5030	398.0	7.9	+2441	+94.3
R. Z.	40	68.2	2974	3205	3078	3103	3090	77.3	2.50	2250	1937	2094	45.2	2.2	− 996	−32.2
J. W.	30	87.8	3272	3302	3413	3161	3287	72.3	2.20	2860	2986	2923	35.7	1.2	− 364	−11.1
G. M.	28	73.0	2465	2426	2447	2445	2445	12.2	0.50	3409	4623	4016	409.0	10.2	+1571	+64.3
A. H.	34	74.1	3374	3074	3323	3125	3224	119.0	3.70	3792	2813	3303	331.0	10.0	+ 79	+ 3.4
G. R. G.	29	63.6	2716	2583	2707	2593	2650	58.3	2.20	3308	3041	3175	90.4	2.8	+ 525	+19.8
L. R.	32	74.5	2911	2881	2927	2865	2896	23.2	0.80	2744	3154	2949	138.0	4.7	+ 153	+ 5.3
H. F.	33	69.1	2451	2551	2441	2562	2501	52.5	2.10	2596	2986	2791	132.0	4.7	+ 290	+11.6
K. F.	29	77.3	3430	3478	3579	3329	3454	86.4	2.50	3629	4272	3951	217.0	5.5	+ 497	+14.4
R. C.	36	81.0	2951	2873	2804	3021	2912	78.6	2.70	3375	3341	3358	11.4	0.4	+ 446	+15.3
J. S.	26	72.7	3124	3211	3315	3023	3169	105.0	3.30	4821	3126	3974	572.0	14.4	+ 805	+25.4
J. W.	30	87.8	3241	3272	3241	3272	3246	17.5	0.54	3069	5921	4495	962.0	21.4	+1149	+35.4
J. C.	49	90.4	4058	4132	4124	4067	4095	31.5	0.77	5720	6617	6168	303.0	4.9	+2073	+50.6
J. G.	30	86.4	3030	3160	3037	3152	3095	58.8	1.90	3750	3750	3750	0.0	0.0	+ 655	+21.2
J. G.	33	68.2	2717	2858	2718	2857	2787	66.9	2.40	5770	4688	5229	366.0	7.0	+2442	+87.6
R. S.	19	75.0	2901	2902	2890	2913	2902	8.7	0.30	3375	4500	3938	379.0	9.6	+2598	+89.5
F. W.	26	68.2	2763	2808	2708	2868	2787	58.5	2.10	3068	3553	3311	163.0	4.9	+ 766	+27.5
D. M.	22	58.2	2356	2434	2592	2199	2395	134.0	5.60	2789	4218	3504	482.0	13.8	+1109	+46.3
M. K. P.	26	63.6	3003	2767	2655	2915	2835	128.0	4.50	3068	3341	3205	92.0	2.9	+ 370	+13.1
R. K.	23	63.6	3060	3181	2976	3265	3121	106.0	3.40	Hemolysis	4383	4383	*	*	+1262	+40.4
P. F.	20	61.4	2672	2950	2756	2866	2811	101.0	3.60	3276	3375	3326	33.0	0.99	+ 515	+18.3
L. J.	19	60.5	2380	2263	2234	2409	2322	69.7	3.00	2410	2812	2611	136.0	5.2	+ 289	+12.4
H. B.	22	50.5	2211	2201	2188	2223	2209	12.1	0.55	2700	2813	2757	38.0	1.4	+ 548	+24.8
D. F.	39	60.9	2326	2474	2302	2498	2400	84.0	3.50	3308	3068	3188	81.0	2.5	+ 788	+32.8
E. B.	24	63.3	2689	2775	2704	2718	2711	29.8	1.10	3629	4066	3848	147.0	3.8	+1137	+41.9
C. K.	24	45.9	2204	2178	2213	2169	2190	16.9	0.77	2596	2557	2577	13.0	0.50	+ 387	+17.7
N. W.	27	61.4	2179	2170	2156	2194	2174	13.0	0.60	2884	3183	3034	101.0	3.3	+ 860	+39.6
V. Mc.	33	59.1	2997	3029	2959	3067	3013	39.2	1.30	4218	4821	4520	203.0	4.5	+1507	+50.0
E. B.	29	57.1	2266	2265	2215	2317	2265	34.0	1.50	1350	Broken tube	1350	*	*	+ 915	+40.0
J. F.	28	65.9	3100	3167	3132	3132	3132	21.9	0.70	Lipemic	3552	3552	*	*	+ 420	+13.4
S. L.	41	69.0	3158	2941	Broken tube	2869	2990	83.7	2.80	3413	3154	3283	87.0	2.65	+ 293	+ 9.8
H. S.	57	60.0	3488	3385	3160	3272	3326	106.0	3.20	4398	4115	4257	96.0	2.3	+ 931	+28.0
G. W.	60	70.5	2832	2816	2790	2858	3282	95.2	2.90	4018	4116	4067	33.0	0.81	+ 785	+23.9
S. R.	23	56.9	2129	2132	2285	1977	2131	104.0	4.90	3068	Not done	3068	*	*	+ 937	+44.0
Average		68.32					2836	58.7	2.30			3558	203.0	5.2	+ 722	+25.5

*Probable error not calculated; single dye determinations only.

of precision of 213 ml., or 5.2 per cent, while the RISA gravimetric method yielded an average probable error of 59 ml., or 2.3 per cent. This difference in probable error is presumed to be due to the differences in accuracy inherent in volumetric and gravimetric measurements, and not to any intrinsic difference in the biochemical or physiologic characteristics of the two substances. RISA is readily adaptable to the gravimetric technique, while the currently available preparations of Evans blue dye must be used volumetrically throughout the procedure.

DISCUSSION

Gregerson[5] discussed the dependability of a solitary plasma volume determination in his discussion of the Army modification of the T-1824 dye method. The problem of complete and uniform distribution of the dye in the body during the interval between injection and sampling as well as the statistical reliability of a single reading is also discussed. The author concluded that no appreciable error is introduced in this manner. The thirty-four simultaneous RISA and Evans blue dye determinations reported in this series of cases would indicate, however, that solitary T-1824 plasma volume determinations as performed routinely by conscientious laboratory technicians show a potential probable error of precision of about plus or minus 10 per cent. The sources of error include: (a) the quantitative delivery of the dye into the vein, (b) the reading of the spectrophotometer, which includes errors due to variations in color in the plasma, hemolysis, lipemia, and the possibility of residual dye in the case of repeated determinations,[10] and (c) each of the two volumetric measurements necessary to prepare the T-1824 dye standard, the control plasma, and the dye-tinged plasma dilutions. Errors introduced in the delivery of the dye into the vein are not likely to exceed 2 per cent. Errors in colorimetry may show wide variations. Under ideal conditions the spectrophotometer should compensate for variations inherent in the color of the plasma and any residual dye; however, these errors may amount to as much as 25 per cent[10] in some cases. Hemolysis and lipemia often make determinations impossible, and results obtained from lipemic or hemoglobin-tinged plasma are most undependable. The actual reading of the spectrophotometer may introduce a small error of 1 or 2 per cent. However, the most significant fact is that volumetric measurements present a large and unpredictable source of possible error. The estimate of 10 per cent over-all potential error in the Evans blue dye method, therefore, is not unreasonable. Zipf, Webber, and Grove[9] have shown that the gravimetric RISA technique presents an over-all probable error of less than 5 per cent.

The clinical use of T-1824 is limited by many factors. Under favorable conditions the use of this blue-azo dye may provide dependable and accurate estimations of blood and plasma volumes; however, favorable or ideal conditions are seldom encountered in clinical use. First, there is universal objection to multiple doses of T-1824 because of the possibility of tinting the skin and mucous membranes of the patient. Although Gregerson[10] has stated that double doses of this dye do not tint the skin, clinicians are generally hesitant about giving more than a single dose of T-1824. Second, the large error which may be introduced

Average 68.32 2836 58.7 2.30 3558 203.0 5.2 + 722 +23.3

*Probable error not calculated; single dye determinations only.

by residual dye in repeated determinations is a technical deterrent to repetition of the test. Clinical application of blood and plasma volume estimations depends upon the dependability of the tests repeated at any time during the course of blood, plasma, or fluid therapy. The limitations of repeated determinations of the T-1824 volume have prevented the extensive use of this method of blood and plasma volume determinations, which offer such great possibility in the definitive control of blood, plasma, and fluid balance in surgery, various medical conditions, and blood dyscrasias. Third, the condition of the patient and of the blood itself may impair the dye determination; the problems introduced by lipemia and hemolysis have already been discussed. Finally, the total of six volumetric measurements incorporated in the dye procedure may produce errors which are clinically as well as technically unacceptable.

The clinical application of RISA as a method of determining blood volume is free of all of the limitations described here for Evans blue dye. The only possible hazard is in repeated doses, which may produce a significant amount of total body radiation. Doses of 20 μc may be repeated as often as five times weekly in a 50-kilogram individual without exceeding the minimum total body radiation of 300 rep per week. Further study may indicate that smaller doses of RISA will be satisfactory. Larger individuals receive correspondingly less total body radiation. Residual radiation from previous determinations is measurable to the same degree of accuracy as the original blood sample. Lipemia, hemolysis, thrombocytopenia, and other variations in the physical and chemical character of the blood and plasma do not alter the potential accuracy of the RISA method. The gravimetric modification[9] of the RISA method eliminates the volumetric errors of other isotope methods as well as the dye methods. The isotope method further offers a means of absolute hematocrit determination free of the errors always present in mechanical centrifugation. In this laboratory the tests performed with RISA and Evans blue dye have shown the almost universal applicability of the isotope method and have illustrated several of the limitations of the dye procedure. It is felt that the superiority of the isotope method should lead to the replacement of the T-1824 method as a standard method of blood and plasma volume determinations.

SUMMARY

The results of thirty-four simultaneous plasma volume estimations with RISA and T-1824 dye are presented and discussed. The gravimetric RISA method appears to have a higher degree of precision as well as a paucity of technical and clinical limitations. The limitations, both clinical and technical, of the T-1824 dye method have prevented its wide clinical application in past years and have led to its replacement in this laboratory by the gravimetric RISA method.

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LABORATORY METHODS

IDENTIFICATION OF BARBITURATES BY ULTRAVIOLET ABSORPTION

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UNTIL the last decade barbiturate analyses generally were performed by means of the colorimetric method introduced by Koppanyi and associates[1] and later modified by Brundage and Gruber[2] and Levy.[3] This test depends upon the formation of a colored complex between the malonyl urea nucleus and cobalt in the presence of isopropyl amine. It suffers from lack of specificity and sensitivity.

Early in the last decade Hellman and associates[4] quantitatively determined Pentothal by means of its ultraviolet absorption in ether solution. Three years later, Jailer and Goldbaum[5] determined Pentothal by means of its ultraviolet absorption in 0.2N sodium hydroxide. Walker and associates[6] reported a method based upon the difference in optical density of the barbiturate in strongly alkaline solution and in weaker alkaline solution caused by the shift in absorption maximum from 255 to 235 millimicrons when the pH is reduced. Goldbaum[7] reported the application of the ultraviolet procedure to extracts of body fluids and tissues and noted the possibility of utilizing the difference in optical density at two different pH levels between 220 and 290 millimicrons as a means of identifying specific barbiturates. Gould and Hine[8] reported a quantitative ultraviolet method which eliminated the blank background always observed in extracts of body fluids and tissues. Finally, Goldbaum[9] described a method for quantitatively identifying a barbiturate based upon the ratios calculated from the optical density differences observed in two different pH environments at certain characteristic wave lengths.

This technique solves the three major drawbacks of the previous methods, i.e., distortion of the absorption curve due to variation in concentration, inability to identify the molecular species of barbiturate specifically, and distortion of the absorption curve due to background interference in extracts from body tissues and fluids. The variation due to concentration is eliminated by setting the maximum optical density difference (at 260 millimicrons for the six barbiturates reported) equal to unity, dividing the optical density differences at all the other wave lengths by this value to obtain the ratios. The distortion due to background interference is overcome by employing the optical density difference ratios instead of the optical density values alone to identify the barbiturate. Since the materials contributing to the background do not change their absorp-

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tion characteristics with a change in pH, they are eliminated. Finally, there is sufficient variation in the optical density difference ratio curves of the barbiturates to make them characteristic.

TABLE I. MELTING POINTS, ULTRAVIOLET ABSORPTION MINIMA AND MAXIMA, AND MAXIMUM POSITIVE OPTICAL DENSITY DIFFERENCE OF 19 BARBITURATES

(Wave lengths are expressed in millimicrons. The maximum positive optical density difference values shown in column 8 represent a concentration of 20 μg/ml. The last column shows the wave length at which the maximum positive difference was observed.)

COMMERCIAL NAME AND SOURCE	DESCRIPTIVE NAME (N.N.R.)	M.P. (° C.)	ULTRAVIOLET ABSORPTION CHARACTERISTICS					
			pH > 11.0		pH 10.4		MAX. POS. O.D. DIFF.	λ MAX. POS. O.D. DIFF.
			λ MAX.	λ MIN.	λ MAX.	λ MIN.		
Alurate (Hoffmann-LaRoche)	5-allyl-5-isopropyl barbituric acid	140-141.5	252	235	240	–	0.446	260
Amytal (Eli Lilly)	5-isoamyl-5-ethyl barbituric acid	155-157	252	232	240	–	0.445	260
Butisol (McNeil)	5-ethyl-5-sec. butyl barbituric acid	165-168	252	232	240	–	0.418	260
Delvinal (Sharp & Dohme)	5-ethyl-5-(1-methyl-1-butenyl) barbituric acid	161-163	252	235	235	–	0.530	260
Dial (Ciba)	5-5-diallyl barbituric acid	171-173	252	235	240	–	0.442	260
Evipal (Winthrop-Stearns)	N-methylcyclohexenyl-methyl barbituric acid	144-145	–	–	–	–	0.055	228
Ipral (E. R. Squibb)	5-ethyl-5-isopropyl barbituric acid	198	252	232	240	–	0.487	260
Luminal (Winthrop-Stearns)	5-ethyl-5-phenyl barbituric acid	170-172	252	235	240	–	0.390	260
Mebaral (Winthrop-Stearns)	5-ethyl-1-methyl-5-phenyl barbituric acid	177-181	252	235	240	–	0.450	260
Nembutal (Abbott)	5-ethyl-5-(1-methyl-butyl) barbituric acid	130-132	252	232	240	–	0.410	260
Neonal (Abbott)	5-N-butyl-5-ethyl barbituric acid	123-124	252	232	240	–	0.470	260
Ortal (Parke,Davis)	5-ethyl-5-hexyl barbituric acid	122-125	252	232	240	–	0.457	260
Pentothal (Abbott)	5-ethyl-5-(methyl-butyl)-2-thio barbituric acid	156-159	305	247	252 305	235 270	0.730	310
Pernoston (Ames)	5-sec. butyl-5-bromallyl barbituric acid	130-133	256	240	240	228	0.322	260
Phanodorn (Winthrop-Stearns)	5-Δ^1 cyclohexenyl-5-ethyl barbituric acid	171-174	254	235	240	–	0.380	260
Sandoptal (Sandoz)	5-allyl-5-isobutyl barbituric acid	138-139	252	235	240	–	0.473	260
Seconal (Eli Lilly)	5-allyl-5-(1-methyl-butyl) barbituric acid	96-100	252	235	240	–	0.413	260
Surital (Parke,Davis)	5-allyl-5-(1-methyl-butyl)-2-thio barbituric acid	130-134	305	247	252 305	270 235	0.461	280
Veronal (Winthrop-Stearns)	Diethyl barbituric acid	191	252	232	240	–	0.480	260

This paper reports our experience with the Goldbaum method over a three-year period and the optical density difference ratios observed at this laboratory for thirteen commercially available barbiturates, in addition to the six reported by Goldbaum.[9]

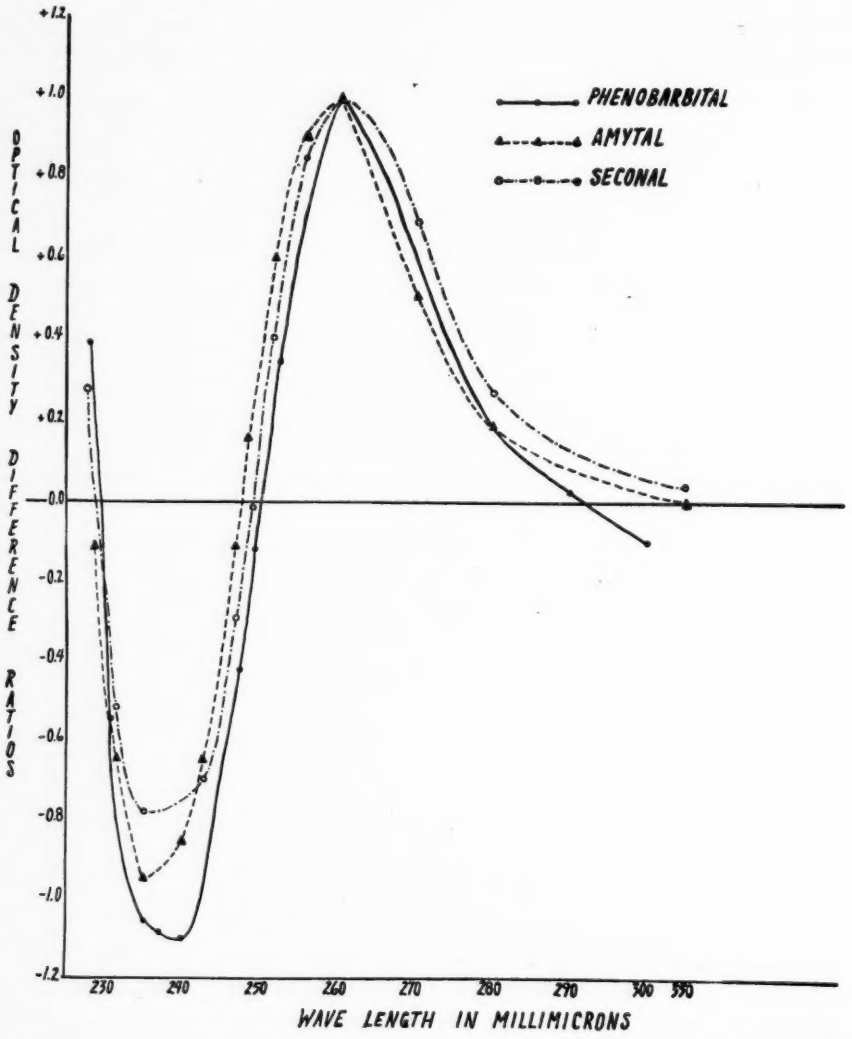

Fig. 1.—Showing optical density difference ratio curves for three typical barbiturates.

METHOD

Since the barbiturates occur in the body as the free acid, it was decided to standardize the procedure against neutral, aqueous solutions of the free barbituric acids, treated as blood in the article by Goldbaum.[9] The free barbituric acids were obtained from the pharmaceutical firms indicated in Column 1 of Table I. The purity was checked by micromelting point determinations, using a Fisher-Johns micromelting point apparatus. When necessary, they were purified by recrystallization from ether solution. Neutral, aqueous solutions were prepared with a concentration of 20 micrograms per milliliter. Five milliliters of these solutions

were transferred to 50 ml., glass-stoppered cylinders containing 40 ml. of chloroform, which had been washed with 0.45N sodium hydroxide. The cylinders were shaken for about three or four minutes, then allowed to stand until the phases separated. The aqueous phase was drawn off and discarded. The organic phase was filtered through coarse filter paper and collected in a clean, dry, 50 ml., glass-stoppered cylinder. Five milliliters of 0.45N sodium

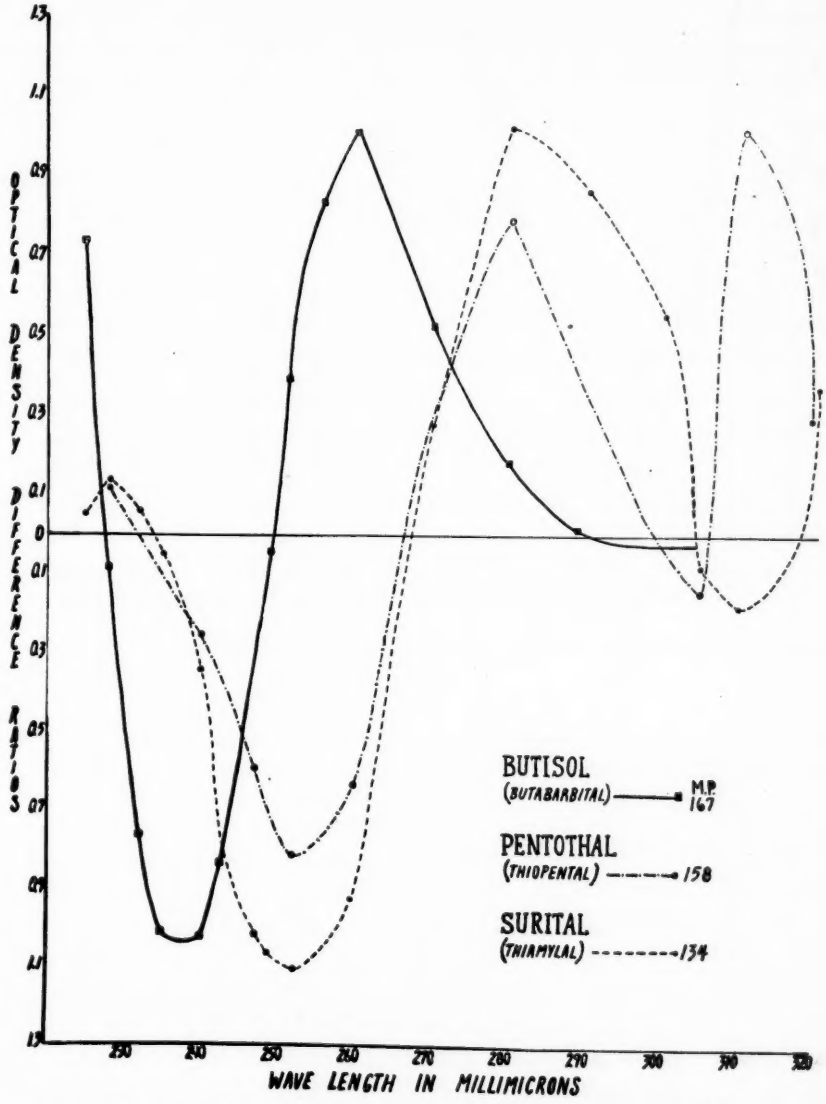

Fig. 2.—Showing the effects of the sulfur on the optical density difference ratio curves. Butisol is included for comparison.

hydroxide was added to the cylinder, which was then shaken for three to four minutes, allowed to stand until the phases separated, after which the aqueous phase was transferred to small, glass-stoppered test tubes and centrifuged for five minutes to remove traces of chloroform.

Approximately 4 ml. of the clear supernatant alkaline solution containing the barbiturate as its sodium salt were transferred to the quartz silica cells and the optical density observed

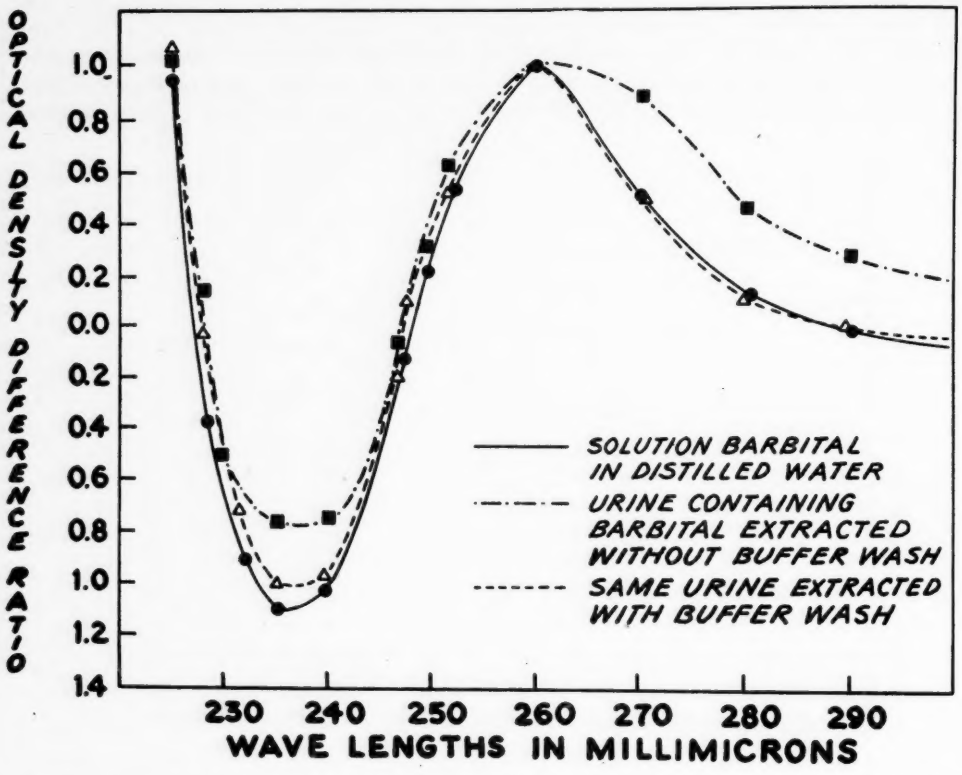

Fig. 3.—Showing the effects of a buffer wash of the chloroform extract on the optical density difference ratio curve.

TABLE II. OPTICAL DENSITY DIFFERENCE RATIOS FOR 19 BARBITURATES

	RATIO =	OPTICAL DENSITY DIFFERENCE / MAXIMUM POSITIVE OPTICAL DENSITY DIFFERENCE											
λ (Mμ)	228	232	235	240	247	249	252	260	270	280	305	310	320
Alurate	+.28	-.56	-.87	-1.0	-.39	-.10	+.10	+1.0	+.68	-	0.0	-	-
Amytal	-.42	-.99	-1.2	-1.1	-.25	+.07	+.53	+1.0	+.52	-	-.05	-	-
Butisol	-.11	-.88	-1.1	-1.2	-.45	-.14	+.34	+1.0	+.63	-	0.0	-	-
Delvinal	+.10	-.58	-.80	-.76	-.11	+.16	+.54	+1.0	+.57	-	0.0	-	-
Dial	+.26	-.60	-.91	-1.0	-.36	-.08	+.37	+1.0	+.63	-	-.03	-	-
Evipal	+1.0	+.78	+.73	+.64	+.44	+.35	+.20	+.018	-.091	-	-.055	-	-
Ipral	-.07	-.74	-.94	-1.0	-.32	-.02	+.41	+1.0	+.64	-	0.0	-	-
Luminal	+.55	-.41	-.78	-.93	-.33	-.06	+.40	+1.0	+.61	-	+.04	-	-
Mebaral	+.02	-.87	-1.0	-1.0	-.29	0.0	+.42	+1.0	+.56	-	-.07	-	-
Nembutal	+.02	-.70	-.96	-1.0	-.32	-.02	+.41	+1.0	+.65	-	-.01	-	-
Neonal	-.23	-.90	-1.1	-1.1	+.28	+.05	+.50	+1.0	+.55	-	0.0	-	-
Ortal	-.13	-.69	-.88	-.84	-.10	+.18	+.59	+1.0	+.56	-	0.0	-	-
Pentothal	+.12	+.05	-.05	-.24	-.59	-.70	-.81	-.62	+.29	+.79	-.14	+1.0	+.29
Pernoston	+1.0	+.06	-.33	-.59	-.30	-.16	+.21	+.81	+.70	+.32	+.07	-	-
Phanodorn	-.72	-1.29	-1.54	-1.47	-.59	-.25	+.27	+1.0	+.51	-	-.08	-	-
Sandoptal	+.19	-.61	-.91	-.96	-.28	-.19	+.45	+1.0	+.60	-	0.0	-	-
Seconal	+.29	-.52	-.78	-.86	-.29	-.02	+.41	+1.0	+.70	-	+.03	-	-
Surital	+.14	+.06	-.05	-.34	-.82	-.96	-1.1	-.92	+.29	+1.0	-.08	-.19	+.37
Veronal	-.26	-.86	-1.1	-.98	-.16	+.14	+.56	+1.0	+.53	-	-.01	-	-

in the Beckman DU Spectrophotometer at the wave lengths indicated in Table II. A blank, obtained by treating 5 ml. of distilled water as the aqueous barbituric acid solutions above, was used as reference. Two milliliters of this alkaline solution were then transferred to small, glass-stoppered test tubes containing 1 ml. of saturated boric acid, mixed by inversion, returned to the clean absorption cells and the optical density at the same wave-lengths as above was observed and recorded.

These values, after correction for the dilution with the boric acid, were subtracted from the optical densities observed at the higher pH. To obtain the characteristic ratios, the optical density difference at each wave length examined was divided by the maximum positive difference.

RESULTS

To avoid the confusion frequently encountered in this field, due to the use of various commercial names, we have included in Table I the most commonly used commercial name as well as the descriptive name. Table I also presents all the maxima and minima observed in the absorption curves in both pH environments, the maximum positive optical density difference which obeys Beer's Law, as shown by Goldbaum,[9] and the wave length at which it was observed.

TABLE III. OPTICAL DENSITY DIFFERENCE RATIOS OBTAINED INDEPENDENTLY BY THREE LABORATORY WORKERS FOR FIVE BARBITURATES

λ (mμ)	228	232	235	240	247	249	252	256	260	270	280	290	305
Amytal													
RFP	−.41	−.87	−1.02	−.94	−.10	+.16	+.60	+.91	+1.0	+.51	–	–	+.01
JRM	−.43	−.98	−1.18	−1.12	−.25	+.07	+.53	–	+1.0	+.52	–	–	−.05
LRG*	−.33	−.88	−1.09	−1.0	−.21	+.12	+.56	–	+1.0	+.52	–	–	–
Nembutal													
RFP	−.17	−.83	−1.05	−1.08	−.37	−.06	+.38	+.84	+1.0	+.63	–	–	+.01
JRM	+.02	−.70	−.96	−1.01	−.32	−.02	+.41	–	+1.0	+.65	–	–	−.01
LRG*	−.15	−.80	−1.0	−1.02	−.38	−.05	+.40	–	+1.0	+.61	–	–	–
Luminal													
RFP	+.52	−.46	−.81	−.94	−.33	−.04	+.39	+.83	+1.0	+.58	–	–	0.0
JRM	+.55	−.41	−.78	−.93	−.33	−.06	+.40	+.84	+1.0	+.61	–	–	−.01
LRG*	+.43	−.48	−.84	−.96	−.36	−.09	+.36	–	+1.0	+.59	–	–	–
Seconal													
RFP	+.29	−.52	−.78	−.86	−.29	−.02	+.41	+.84	+1.0	+.70	–	–	+.03
LRG*	+.21	−.63	−.89	−1.04	−.39	−.13	+.30	–	+1.0	+.69	–	–	–
Butisol													
RFP	0.0	−.73	−.95	−1.0	−.37	−.06	+.38	+.82	+1.0	+.65	+.21	+.04	0.0
RFP	−.08	−.77	−1.01	−1.02	−.33	−.04	+.39	+.83	+1.0	+.63	+.18	+.01	−.02

*From Goldbaum.[9]

TABLE IV. EFFECTS OF ENTRAINED CHLOROFORM ON THE ULTRAVIOLET ABSORPTION OF 0.45N SODIUM HYDROXIDE

STANDING TIME IN MINUTES	WAVE LENGTH IN MILLIMICRONS													
	225	228	232	235	240	247	249	252	256	260	270	280	290	305
15	.259	.173	.108	.086	.068	.051	.042	.036	.026	.022	.018	.018	.017	.016
30	.245	.158	.100	.077	.062	.046	.040	.033	.024	.018	.016	.016	.016	.014
45	.230	.136	.097	.076	.061	.044	.038	.031	.024	.018	.016	.015	.015	.013
60	.220	.125	.094	.075	.060	.043	.037	.030	.024	.018	.016	.015	.015	.013
120	.176	.121	.082	.073	.058	.040	.035	.027	.022	.017	.014	.013	.013	.012
480	.110	.093	.072	.069	.058	.040	.035	.029	.023	.018	.014	.013	.013	.012

TABLE V. REVIEW OF BARBITURATES QUANTITATIVELY IDENTIFIED BY MEANS OF THE GOLDBAUM ULTRAVIOLET METHOD[9] OVER A THREE-YEAR PERIOD AT THIS LABORATORY

CASE NO.	BARBITURATE	CONC. IN μG/ML. UNLESS OTHERWISE INDICATED	SPECIMEN	REMARKS
1A	Amytal	9.7	Blood	Autopsy specimen. Pt. died in diabetic coma. Received 1½ grains Luminal, 7½ grains Amytal 10 hr. before death
1B	Amytal	18.0	Urine	
2A	Amytal	3.5	Blood	Pt. received 487 mg. I.V. 3 hr. before blood drawn. Urine collected from 3rd to 7th hr. after injection
2B	Amytal	16.7	Urine	
3	Amytal	7.2	Blood	
4	Amytal	4.1	Blood	U. V. analysis confirmed by mixed m.p. on crystals recovered by vacuum microsublimation
5	Amytal	9.1	Blood	
6A	Amytal	53.0	Urine	Duplicate analyses run on two aliquots
6B	Amytal	50.0		
7A	Luminal	4.1	Blood	
7B	Luminal	14.0	Urine	
8A	Luminal	26.6	Blood	Samples C, D, E, and F were serial washings from stomach
8B	Luminal	19.6	Urine	
8C	Luminal	268.0	St. wash.	
8D	Luminal	15.7	St. wash.	
8E	Luminal	9.1	St. wash.	
8F	Luminal	6.7	St. wash.	
9	Luminal	16.0 mg./tablet	Tablet	
10	Luminal	18.5	Blood	
11	Luminal	880.0	St. wash.	
12A	Luminal	12.5	St. wash.	Tablet was found in pt. possession
12B	Luminal	12.4 mg./tablet	Tablet	
13	Luminal	19.8	Blood	Specimen taken at autopsy. Pt. rec'd 2 grains Luminal 9½ hr. before death
14	Luminal	15.0 mg./tablet	Tablet	Confirmed by m.p. on sublimate
15	Luminal	19.5	Blood	
16	Luminal	24.5	Urine	Unable to determine m.p. of sublimate accurately
17	Luminal	107.0	Urine	
18	Mebaral	138.0	Urine	
19	Nembutal	60.0	St. wash.	
20	Nembutal	14.5	Blood	
21	Nembutal	65.0	Urine	
22A	Neonal	73.0 μg/Gm.	Brain	Suicide. Victim ingested 105 gr. of barbiturate. Analyses confirmed by m.p. on sublimate from blood
22B	Neonal	58.5 μg/Gm.	Kidney	
22C	Neonal	180.0	Blood from kidney	
23	Neonal	96.0 mg./capsule	Capsule	Confirmed by m.p. on residue from acid aqueous extract
24A	Pernoston	4.8	Serum	
24B	Pernoston	4.5	Urine	
24C	Pernoston	3.6	St. wash.	
25A	Seconal	7.4	Blood	Attempted suicide. Analyses confirmed by m.p. on sublimate from blood extract. Tablet and capsule found on pt. identified as Neonal and Nembutal, respectively
25B	Seconal	6.0	Urine	
25C	Seconal	86.0	St. wash.	
26	Seconal	96.0 mg./capsule	Capsule	
27	Seconal	70.0	Blood	

TABLE V. CONT'D

28A	Seconal	30.0		Urine	Ratio curve from blood extract did not fit any standard. Appeared to be mixture of two or more drugs
28B	Nembutal?	7.3		Blood	
29	Seconal	27.5		Blood	
30A	Seconal	37.5		Blood from liver	Specimens taken at autopsy
30B	Seconal	676.0		St. contents	
31	Seconal	96.0	mg./capsule	Capsule	
32A	Seconal	2.5		Serum	
32B	Seconal	10.3		Urine	
33A	Seconal	9.2		Blood	
33B	Seconal	45.0		Urine	

U.V. = Ultraviolet; m.p. = melting point; St. wash. = stomach washings.

It can be seen from Table I that Evipal was the only barbiturate examined which did not show the characteristic absorption. This is probably due to the hexenyl ring which hinders the resonance in the malonyl urea nucleus.

Fig. 1 shows the shape of the optical density difference ratio curves when plotted on linear graph paper. Fig. 2 shows the shift of the wave length of maximum optical density difference toward the longer wave lengths in the case of the thiobarbiturates. The curve for Butisol is included for comparison. Figs. 1 and 2, and the data in Table II, show that the optical density difference ratios are truly characteristic for the barbiturates investigated. Fig. 3 confirms Goldbaum's observation that chloroform extracts from urine in his procedure must be washed with an aqueous, 1M phosphate buffer solution with a pH of 7.4 to rid them of interfering substances. Urine was found to give the greatest difficulty of all biologic material investigated.

The barbiturates are unstable in alkaline solution and cannot be stored for more than an hour or so in such solutions. However, we observed no appreciable loss of barbital stored in chloroform up to 24 hours. At the end of 96 hours of storage there was a loss of 21 per cent.

Table III reflects the reproducibility of the method by comparing the optical density difference ratios observed by the authors and by Goldbaum.[9] These data were for aqueous solutions of the free barbituric acids treated as the standards described above.

To determine the effect of chloroform not removed by centrifugation, we shook 50 ml. of chloroform with 5 ml. of 0.45N sodium hydroxide for four minutes, allowed the phases to separate, transferred the aqueous phase to a test tube, centrifuged and then observed the optical density when read against untreated sodium hydroxide at the wave lengths and time intervals indicated in Table IV. These data emphasize the necessity of preparing a reagent blank each time an analysis is performed.

When 5 ml. of human blood serum containing 16 micrograms of barbital per milliliter was extracted at a pH of 7.0, only 65 per cent was recovered, while 86 per cent was recovered when the pH was lowered to 2.5.

TABLE VI. OPTICAL DENSITY DIFFERENCE RATIOS OBSERVED FOR THE CASES DESCRIBED IN TABLE V

	WAVE LENGTH IN MILLIMICRONS													
CASE NO.	228	232	235	240	247	249	252	256	260	270	280	290	305	
1A	+1.01	−0.09	−0.23	−0.44	−0.08	+0.23	+0.54	+0.95	+1.0	+0.58	+0.37	+0.24	−0.11	
1B	+0.04	−0.44	−0.69	−0.65	−0.25	+0.06	+0.52	+0.96	+1.0	+0.56	+0.14	−0.01	+0.02	
2A	−0.07	−1.24	−1.64	−1.67	−0.48	−0.15	+0.33	+0.88	+1.0	+0.37	+0.04	−0.07	−0.11	
2B	−0.23	−0.06	−0.72	−0.68	−0.06	+0.15	+0.54	+0.92	+1.0	+0.63	+0.15	+0.08	+0.18	
3	−0.60	−0.99	−1.4	−1.61	−0.86	−0.46	+0.06	–	+1.0	+0.51	–	–	−0.09	Amytal
4	−1.95	+0.40	+0.16	−0.45	−0.03	–	+0.52	–	+1.0	+0.71	–	–	+0.11	
5	−2.03	−1.53	−1.12	−0.93	−0.14	+0.12	+0.53	+0.83	+1.0	+0.52	+0.19	+0.09	+0.02	
6A	−0.14	−0.14	−0.21	−0.67	0.0	+0.14	+0.63	–	+1.0	+0.53	–	–	+0.30	
6B	−0.93	−1.43	−1.43	−1.60	−0.50	−0.13	+0.36	–	+1.0	+0.56	–	–	+0.27	
7A	+1.0	−0.67	−1.14	−1.42	−0.76	−0.43	+0.13	–	+1.0	+0.34	–	–	−0.19	
7B	+0.91	−0.16	−0.61	−0.75	−0.21	+0.01	+0.39	–	+1.0	+0.48	–	–	+0.03	
8A	+0.59	−0.46	−0.82	−1.0	−0.43	−0.11	+0.33	–	+1.0	+0.60	–	–	+0.01	
8B	+0.54	−0.47	−0.82	−0.95	−0.39	−0.10	+0.34	–	+1.0	+0.60	–	–	+0.01	
8C	+0.40	−0.74	−1.09	−1.22	−0.52	−0.17	+0.32	–	+1.0	+0.58	–	–	+0.07	
8D	+1.11	+0.22	−0.04	−0.16	+0.16	+0.33	+0.64	–	+1.0	+0.65	–	–	+0.15	
8E	+0.67	−0.22	−0.51	−0.65	−0.01	+0.05	+0.39	–	+1.0	+0.59	–	–	+0.10	
8F	+1.2	+0.21	−0.13	−0.40	−0.06	+0.18	+0.50	–	+1.0	+0.72	–	–	+0.17	
9	+0.43	−0.55	−0.89	−1.04	−0.45	−0.15	+0.32	+0.81	+1.0	+0.06	+0.18	+0.03	−0.02	Luminal
10	+0.50	−0.65	−0.96	−1.17	−0.54	−0.22	+0.28	+0.78	+1.0	+0.56	+0.13	+0.01	−0.04	
11	+0.21	−0.54	−0.86	−0.97	−0.35	−0.02	+0.42	+0.87	+1.0	+0.62	+0.16	–	–	
12A	+0.62	−0.31	−0.58	−0.84	−0.30	−0.07	+0.35	–	+1.0	+0.58	–	+0.06	–	
12B	+0.25	−0.51	−0.79	−0.90	−0.30	−0.06	+0.26	–	+1.0	+0.58	–	+0.05	–	
13	+0.32	−0.72	−1.04	−1.13	−0.48	−0.16	+0.31	+0.82	+1.0	+0.61	+0.16	+0.03	−0.01	
14	+0.33	−0.75	−1.15	−1.29	−0.62	−0.39	+0.26	–	+1.0	+0.58	–	–	−0.09	
15	+0.58	−0.56	−0.84	−1.04	−0.47	−0.18	+0.32	–	+1.0	+0.59	+0.17	+0.03	−0.02	
16	+0.92	0.0	−0.29	−0.40	+0.03	+0.24	+0.57	–	+1.0	+0.58	–	–	+0.09	
17	+0.50	−0.51	−0.81	−0.94	−0.34	−0.07	+0.39	+0.81	+1.0	+0.59	+0.19	+0.07	+0.001	
18	−0.20	−0.44	−0.32	−0.33	+0.16	+0.23	+0.55	–	+1.0	+0.39	–	–	−1.17	Mebaral
19	−0.07	−0.78	−0.98	−1.02	−0.36	−0.06	+0.38	–	+1.0	+0.59	–	–	+0.03	
20	−0.26	−0.98	−1.3	−1.4	−0.58	−0.19	+0.34	+0.85	+1.0	+0.64	+0.18	+0.02	−0.02	Nembutal
21	−0.19	−0.77	−0.98	−1.02	−0.33	−0.04	+0.40	–	+1.0	+0.62	–	+0.08	–	
22A	−0.33	−0.80	−0.95	−0.93	−0.28	0.0	+0.33	–	+1.0	+0.52	–	–	+0.05	
22B	−0.34	−0.98	−1.22	−1.22	−0.44	−0.20	+0.29	–	+1.0	+0.67	–	–	+0.01	Neonal
22C	+0.20	−0.64	−0.95	−1.03	−0.39	−0.10	+0.37	–	+1.0	+0.65	–	–	+0.01	
23	+0.07	−0.54	−0.78	−0.78	−0.21	+0.07	+0.45	+0.84	+1.0	+0.66	+0.29	–	–	

24A	+1.0	+0.19	−0.13	−0.35	−0.23	−0.06	+0.10	–	+0.28	+0.08	–	–	−0.08	
24B	+1.0	+0.05	−0.27	−0.38	−0.15	−0.04	+0.23	–	+0.56	+0.32	–	–	−0.08	Pernoston
24C	+1.0	+0.26	−0.02	−0.13	−0.02	+0.06	+0.19	–	+0.32	+0.23	–	–	−0.01	
25A	+1.0	−0.21	−0.67	−0.94	−0.39	−0.15	+0.26	–	+1.0	+0.60	–	–	+0.03	
25B	+1.0	−0.08	−0.27	−0.31	−0.15	+0.06	+0.44	–	+1.0	+0.41	–	–	−0.01	
25C	+0.03	−0.70	−1.0	−1.13	−0.51	−0.21	+0.28	–	+1.0	+0.68	–	–	+0.04	
26	+0.33	−0.42	−0.72	−0.78	−0.27	0.0	+0.36	+0.79	+1.0	+0.72	+0.33	–	–	
27	+0.52	−0.53	−0.90	−1.04	−0.42	−0.11	+0.34	–	+1.0	+0.58	–	−0.03	–	
28A	+0.18	−0.79	−1.11	−1.15	−0.48	−0.12	+0.31	–	+1.0	+0.56	–	–	–	
28B	−0.28	−1.78	−2.33	−2.5	−1.46	−0.96	−0.21	–	+1.0	+0.30	–	–	–	
29	+0.20	−1.0	−1.45	−1.56	−0.81	−0.44	+0.13	–	+1.0	+0.54	–	–	–	Seconal
30A	+0.23	−1.09	−1.6	−1.71	−0.99	−0.63	+0.08	–	+1.0	+0.53	–	–	–	
30B	+0.28	−0.25	−0.96	−1.10	−0.52	−0.34	+0.50	–	+1.0	+0.70	–	–	–	
31	+0.29	−0.65	−0.99	−1.10	−0.49	−0.20	+0.28	–	+1.0	+0.71	–	–	–	
32A	–	−0.17	−0.90	−1.1	−0.47	+0.02	–	+0.69	+1.0	+0.71	–	–	−0.02	
32B	–	−0.08	−0.81	−0.95	−0.38	+0.05	–	+0.71	+1.0	+0.65	–	–	−0.01	
33A	+0.83	−0.19	−0.61	−0.92	−0.28	+0.07	+0.45	+0.85	+1.0	+0.57	+0.34	+0.26	+0.08	
33B	−0.27	−1.46	−1.90	−1.92	−0.86	−0.57	+0.30	+0.87	+1.0	+0.45	+0.28	+0.06	−0.17	

This is not surprising, since the pK for barbital is 7.89, according to Bush.[10] This same author reports the pK values for four other barbiturates as follows: phenobarbital 7.34, Amytal 7.89, pentobarbital 8.04, and Evipal 8.23. Goldbaum[9] objects to the use of a more acid pH in the aqueous phase because of the possibility of extracting other acidic drugs, such as salicylic acid, which would interfere with the ultraviolet absorption.

We were able to confirm the fact that salicylic acid, if present, would interfere with the procedure as described. However, the secondary maximum of this compound at 298 millimicrons is great enough to make its presence known in such a case. A later article by Goldbaum,[11] in which he shows that a considerable fraction of the barbiturate is bound to albumin at the pH of blood while considerably less is so bound at lower pH values, raises some question as to the advisability of extracting for barbiturates at the neutral pH.

Currently, Brackett and Braford[12] are employing a pH of 4.5 to 5.0 for extraction of acidic drugs from biologic material. We are investigating this matter of the ideal pH for routine toxicological analyses for barbiturates in more detail at present in this laboratory.

Table V and Table VI present the results of our positive findings in barbiturate analyses, using this method over the past three years. Only in Cases 1, 2, and 13 was the nature of the barbiturate known in advance of the analysis. We have found it helpful to observe the optical density differences at 256, 280, 290, and 305 millimicrons in addition to the wave lengths employed by Goldbaum.[9] We have also found it helpful in doubtful cases to plot the ratio curve observed on transparent "onion skin" paper, which can then be superimposed on standard curves to find the best match. Whenever possible, it is recommended that the results of the ultraviolet absorption studies be confirmed by melting point determinations on the crystals recovered by vacuum sublimation of the residue from an ether extraction of the sodium hydroxide solution after acidification with concentrated acid. The microsublimation apparatus described by Maher[13] has proved quite helpful in this operation.

Although the data in Table VI show that this method is not ideal for routine toxicological analysis, it is the opinion of the authors that it is the best method thus far described for the identification of barbiturates by ultraviolet absorption.

SUMMARY

The ultraviolet absorption method of Goldbaum[9] for the identification and estimation of barbiturates in biologic tissues and fluids has been investigated. The optical density difference ratios for 13 commercially available barbiturates, in addition to the six reported by Goldbaum,[9] are presented. Our experience with this procedure in routine toxicological analyses over a three-year period is summarized.

We wish to express our gratitude to the pharmaceutical manufacturers listed in Column 1 of Table I for their kind donation of the barbituric acids used in this study.

We are also indebted to Captain Robert S. Cox, MC, USA, for technical assistance in completing this work.

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MICRODETERMINATION OF CORTICOSTEROIDS WITH TETRAZOLIUM DERIVATIVES*

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INTRODUCTION

ALL the known biologically active corticosteroids possess a primary α-ketol group. Such compounds are reducing agents. Chen and collaborators[3] and also Mader and Buck[1] have applied this fact to the determination of these steroids by the use of tetrazolium derivatives. Salts like 2,3,5-triphenyltetrazolium chloride (TPTZ) and 3,3′-dianisole bis-4,4′ (3,5-diphenyl) tetrazolium chloride (blue tetrazolium, BT) were found easily reduced to colored formazans in the presence of these corticosteroids.[1-3] The total or partial reduction of BT is dependent on two factors: the temperature at which the reaction is carried out and the pH of the medium.[3] The total reduction of BT is manifested by the formation of a blue-mauve color, while a pink color is produced when only one-half of the BT molecule is reduced. We have studied these procedures in an attempt to improve the sensitivity of these reactions.

EXPERIMENTAL

(A) *Qualitative Test Applied to Paper Chromatography.—*

Reagents:

1. Solutions of 10 per cent and 20 per cent sodium hydroxide in 60 per cent methanol (V/V).

2. Aqueous solutions of:

500 mg. per cent BT,
200 mg. per cent TPTZ,
200 mg. per cent BDT (3-o-diphenyl-2,5-diphenyl tetrazolium chloride),
200 mg. per cent TDT (3-o-tolyl-2,5-diphenyl tetrazolium chloride).

One part BT and three parts NaOH solutions are mixed just before use. The reagent is sprayed on the dried chromatograms containing the corticosteroids or other steroids with a reducing group. When the 10 per cent NaOH solution is used, a maximum blue-mauve color intensity is developed in 15 minutes and as little as 0.25 μg of steroid per sq. cm. can be detected. With the 20 per cent NaOH solution as little as 0.05 μg of steroid per sq. cm. can be detected. But, in this latter case, it is necessary to immerse the paper strip in 2N sulfuric acid within 6 to 7 minutes after spraying in order to avoid darkening of the background and destruction of the paper. If the dried paper strip is finally sprayed with acetone-water solution (70 per cent V/V), the color intensity of the spots on the paper chromatogram is slightly increased. (For quantitative purposes, the "colored spots" can be eluted with 70 per cent aqueous acetone [V/V] and colorimetric determinations can be made.)

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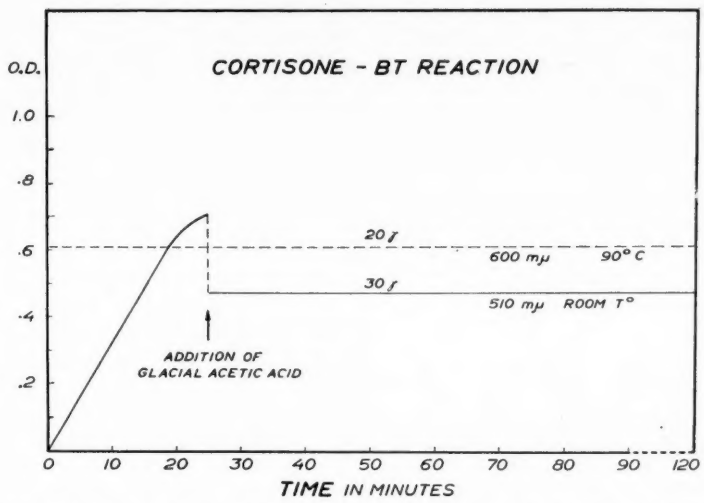

Fig. 1.—Stability of the color reactions obtained at 90° C. and at room temperature with cortisone. The lower curve, which represents the reaction carried out at room temperature, is started after the period of 25 minutes, at which time the glacial acetic acid is added to arrest the reaction.

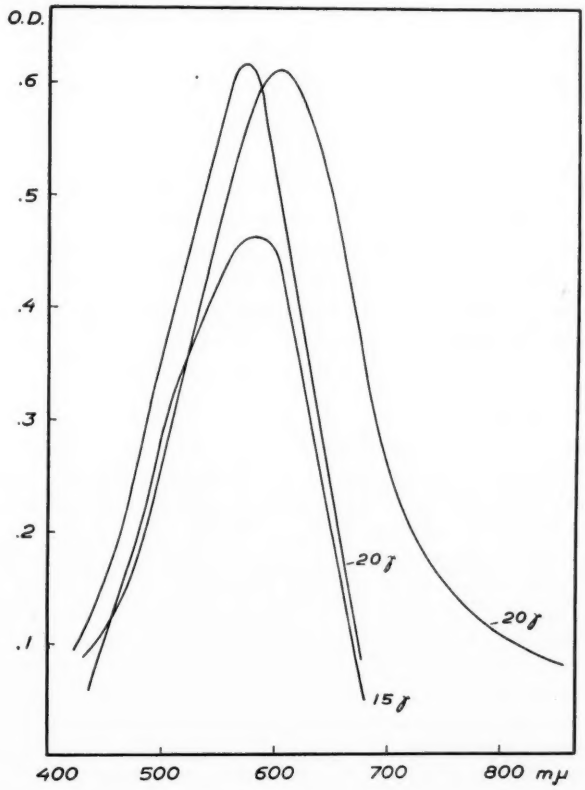

Fig. 2.—The absorption maxima of the curve obtained when the reaction is carried out at 90° C. may vary between 580 and 600 mμ, depending on the purity of the batch of BT used. When the reaction is carried out at room temperature, the absorption maxima may vary between 510 and 525 mμ.

Using TPTZ with 10 per cent and 20 per cent sodium hydroxide, as little as 5 μg and 2 μg of corticosteroids per sq. cm., respectively, can be detected under the same conditions. With both BT and TPTZ, equal concentration of 11-dehydrocorticosterone,* 17-hydroxycorticosterone, 17-hydroxy-11-desoxycorticosterone, 11-desoxycorticosterone develop the same color intensities, while with corticosterone and 17-hydroxy-11-dehydro-corticosterone, the intensities are slightly less. We have found that the acetate of Compound A (the only one which was tried) developed its color at a slower rate than the free compound. If the solutions of BDT and TDT are used under the same conditions, the intensity of violet-red color is about 4 times less than with BT. In all cases, use of a more dilute solution of methanol or its replacement by ethanol only serves to decrease the sensitivity of the reaction.

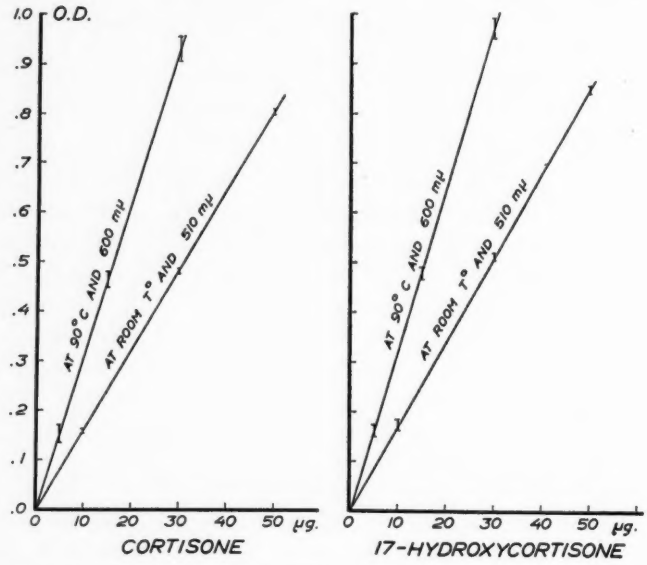

Fig. 3.—Optical densities obtained with cortisone and 17-hydroxycortisone at 600 mμ when the reaction is carried out at 90° C. and 510 mμ when the reaction is carried out at room temperature.

The vertical lines on the cortisone curves represent the range of variations given by 10 different determinations and those on the 17-hydroxycortisone curves represent the range of variations of 6 different experiments.

(B) *Quantitative Test.—*

Reaction carried out at 90° C.:

Reagents (should be prepared immediately before use):

1. Buffered BT solution: 50 mg. of BT is dissolved in 9 ml. of ethanol to which is added 11 ml. of 10 per cent aqueous tetramethylammonium hydroxide solution. The resulting solution has a pH of 12.5 and contains 2.5 mg. of BT per milliliter.

2. Acetic acid solution made of one part glacial acetic acid plus two parts of water.

3. Cortisone in 5, 15, and 30 μg amounts as standards.

Into 5 ml. test tubes is placed 0.4 ml. of the buffered BT solution followed by 0.5 ml. of water. The tubes are heated in a small flame without stirring. It is very important not to allow boiling or agitation of the solution. If the color remains, the addition of 1 drop of water is usually sufficient to make it disappear. The colorless warm reaction mixtures are poured into 10 ml. test tubes containing the dry corticosteroid residue. The tubes are then heated without stirring in the absence of light for 2 minutes in a water bath kept at 90° C. The solutions are immediately cooled and neutralized by the addition of 0.1 ml. acetic acid solution. The neutralized solutions are well mixed and 2 ml. of A.R. acetone is added to

*The corticosteroids used in this investigation were kindly supplied to us by Dr. Fred Wrigley of the Ciba Company and by Dr. John Laurie of the Merck Company.

dissolve the formazan produced in the reaction. The final mixtures, blue-mauve in color, are transferred to 4 ml. Corex cuvettes and the optical densities read at 600 mμ in a Beckman Quartz Spectrophotometer, model DU (see legend, Fig. 3). The blank is prepared in the same manner as the final mixtures except without cortisone. The cuvettes are covered during the readings in order to prevent the evaporation of the acetone and the precipitation of the formazan. The color remains stable for at least 2 hours (Fig. 1). The sensitivity of the reaction can be further increased by the use of microcuvettes.

Reaction carried out at room temperature:

Reagents:

1. Tetramethylammonium hydroxide solution prepared by adding 1 ml. of 10 per cent aqueous tetramethylammonium hydroxide to 9 ml. of 95 per cent ethanol.
2. BT reagent prepared by dissolving 50 mg. of BT in 10 ml. of 95 per cent ethanol.
3. Cortisone in 10, 30, and 50 μg amounts as standards.

All the reagents should be prepared immediately before use.

The cortisone standards are placed in test tubes and 1.5 ml. of distilled 95 per cent ethanol is added, followed by 0.25 ml. of tetramethylammonium hydroxide solution. Then 0.25 ml. of BT reagent is added. The resulting solutions are well mixed and acidified after a period of 25 minutes by the addition of 1 ml. of glacial acetic acid. The final solutions, pink in color and at about pH 3, are again well mixed and transferred to 4 ml. Beckman Corex cuvettes. The color intensities are read at 510 mμ (see legend, Fig. 3), in the Beckman Quartz Spectrophotometer. The acidification arrests the color reaction, which remains stable for at least 2 hours (Fig. 1). The blank, which remains uncolored, is prepared in the same manner described previously except that no cortisone is added.

DISCUSSION

The tetrazolium salt solutions are photosensitive and should be prepared daily, except for the TPTZ solution, which can be stored in a dark bottle. The colored formazans resulting from the reduction of the tetrazolium salts by corticosteroids are also photosensitive, and it is well to carry out the quantitative determinations in the absence of light. The BT compound is obtainable in different degrees of purity. For each sample used, the absorption maxima may vary within 20 mμ and the optical densities will also vary slightly. Therefore, standard curves should be established for each batch of BT (Fig. 2). The curves obtained with the optical densities versus concentration of corticosteroids follow Lambert-Beer's law, as illustrated in Fig. 3.

CONCLUSIONS

Qualitative tests as applied to paper chromatography for the identification of corticosteroids by the use of tetrazolium derivatives have shown blue tetrazolium (BT) to be the most satisfactory agent.

Procedures which are used for the quantitative determination of corticosteroids by the reduction of BT have been modified in order to increase the sensitivity of the reaction and the stability of the color produced. This reaction is seen to depend on the temperature and the pH at which it is carried out.

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A SIMPLE WARBURG CALIBRATOR*

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In 1949, Lazarow[1] published a description of a micrometer calibrator for Warburg manometers using a volumetric method. This method was modified[2] so that the volume of the entire system, manometer plus vessel, could be calibrated in a single operation. The newer method required an adapter and a special clamp for holding the apparatus on the manometer. This paper describes the design of a calibrator which is simpler to manipulate and permits the very rapid calibration of a set of manometers and vessels.

The apparatus is illustrated in Fig. 1. It consists of a 25 × 100 mm. Lucite rod (*F*) threaded to fit the metal micrometer portion of the Lazarow apparatus (*A*) when the usual plastic adapter is removed.† A 7/16 inch hole 1¼ inches deep (*D*) is bored into the rod to accommodate the plunger of the micrometer. A small groove is cut into the side of this hole with a boring tool to take an "O" ring (*B*), which makes a water-tight seal with the plunger. The "O" ring can be removed from the adapter with which the micrometer comes equipped. At the opposite end of the unit a ⅝ inch hole (*H*) was bored. The bottom of the hole was squared with a boring tool. The depth of the hole is not critical so long as it is not greater than the length of capillary projecting above the stopcocks of the manometers to be calibrated. A 3/32 inch capillary (*E*) is now drilled to connect the two ends of the apparatus, and the entire assembly is polished with jeweler's rouge until all surfaces are transparent. A ¼ inch length of pressure tubing (¾ inch diameter × ¼ inch hole) (*G*) is now forced into (*H*) to provide a tight fit between manometer and calibrator.

The calibration procedure has been modified from that of Lazarow.[2] The chamber (*D*) and the capillary (*E*) are one-half filled with water, and all air bubbles are removed by rapidly moving the plunger up and down. The plunger is backed off until the water level is one-half way up the capillary and the micrometer is about one-half open. It is convenient to have the entire Warburg assembly in a water bath at room temperature. The apparatus is now forced onto the capillary above the stopcock on the manometer. The rubber tubing (*G*) serves as a source of support and to make an air-tight connection. During this operation the vent on the Warburg vessel should be open.

The column of water in the capillary is advanced by turning the micrometer screw until the manometer capillary and the stopcock bore are just filled with

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†The micrometer and adapter of Lazarow[2] are built by the Micro-Metric Instrument Co., Cleveland, Ohio, and are available through scientific supply houses.

liquid. The manometer is adjusted to 150 mm. (or any convenient point) and the vent to the vessel is closed. Both arms of the manometer should read the same. With the system in equilibrium, read the micrometer. Proceed to back

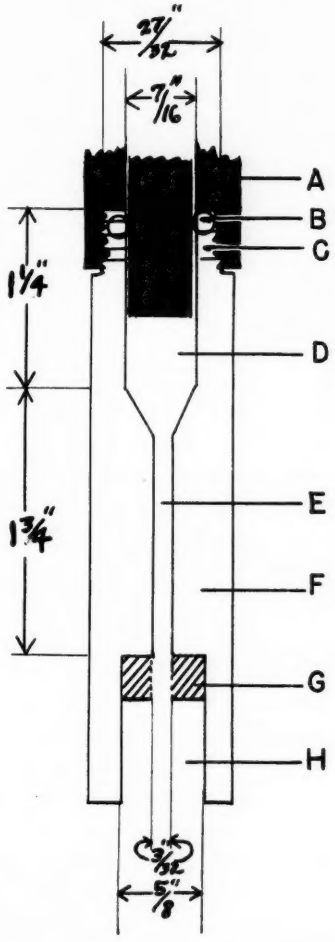

Fig. 1.—A simplified Warburg calibrator. Dimensions and materials are indicated in the text. The solid black portion of the drawing represents the plunger and other metallic parts of the Lazarow calibrator as built by the Micro-Metric Company.

off the micrometer, adjusting the closed arm to 150 mm. until the difference in the two manometers' arms (p) is equal to:

$$p = 0.129\ (P_a - P_w)^*$$

where P_a is the corrected atmospheric pressure and P_w is the vapor pressure of water at the temperature of calibration. The open arm of the manometer is set to 150-p with the closed arm at 150 mm. at the end point of calibration.

*The theoretical considerations leading to the development of the equation have been discussed by Lazarow.[2]

At the end point, the micrometer setting is again read. The difference in the initial and final readings multiplied by ten is equal to the volume of the system in milliliters. If the water level is returned to the original position in the bore of the stopcock and the manometer adjusted to 150 mm. on the closed side, both arms should read 150 mm. and the micrometer reading should be the initial reading. If this is not the case, a leak exists in the system.

The proposed method is more adaptable than that of Scholander,[3] for it permits the calibration of any size vessel, and direct readings of volume are obtained without elaborate calculation.

Using the apparatus as described we have calibrated several sets of manometers and vessels. In every instance, 5 to 10 separate calibrations on a single system have given volumes within 0.7 per cent of each other. Three workers calibrating the same set of manometers have agreed within 0.6 per cent, and volumes of manometers and flasks determined by this method and compared to volumes of the same vessels determined in another laboratory by the mercury method agreed within 0.4 per cent.

We wish to express special appreciation to Mr. Harold Breeding, Machinist, Department of Physiology, who built this apparatus.

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AN APPARATUS FOR MEASUREMENT OF ACTIVITY IN SMALL ANIMALS

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THE diffuse activity of animals has provided an index of behavior useful to both the physiologist and the psychologist. Since the early work of Szymanski,[1-3] various modifications of tambour- and spring-suspended cages have been used to assay diffuse activity in studies of animal behavior[4-8] and the influence upon behavior of illumination,[4, 6, 9] cerebral lesions,[10-13] drugs,[14-18] and adrenalectomy.[19] In addition, tambour- or spring-suspended cages have been utilized in studies of audioconvulsive reactions, audiosensitivity,[11] and a conflict situation.[20] Boxes equipped with photocell-activated counters have been used to study the effects of pharmacologic agents[21] and of confinement[22-24] upon diffuse activity. Another type of box utilizing a multiple-section floor with electrical contacts under each floor section was used to determine the effect of radioactive pellets on the normal activity of animals.[25] A cage utilizing a capacitance antenna was developed to determine the effects of forced exercise and the effects of nutrition upon diffuse activity.[26] Recently an electronic integrator unit was designed for measuring the diffuse activity of animals.[27]

A study of diffuse activity was considered pertinent to an investigation of the effects of irradiation upon animal performance. A relatively large number of activity units were deemed necessary for statistically reliable results because of the anticipated wide range of daily activity among animals. Because 60 to 70 days were required for the reattainment by irradiated rats of activity comparable to that of control animals in behavior studies utilizing wheel cages,[28] it was expected that the diffuse activity units would be in continuous use for a similar period of time.

With these considerations in mind, the literature was searched for a cage-recording unit that would (1) be reasonably sensitive, yet stable enough for long-term continuous usage, (2) require minimum maintenance with respect to mechanical adjustments and to food and water replenishment in order to prevent undue disturbance of the animals, and (3) provide information concerning activity in an integrated, readily usable form in order to facilitate data analysis. In addition, a method of calibration appeared essential in order to determine the stability of the unit over varying periods of time.

The difficulty in evaluating kymographic data for large numbers of animals over relatively long periods of time negated the use of the units described in the

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The data here given will form a portion of the material to be submitted in candidacy for the M.S. degree at the University of San Francisco by T. J. C.

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literature which utilized only simple graphic recording devices. A modified form of the electronic unit described by Campbell and McLean[27] was tested, but was found to give inconsistent results from day to day with respect to a standard

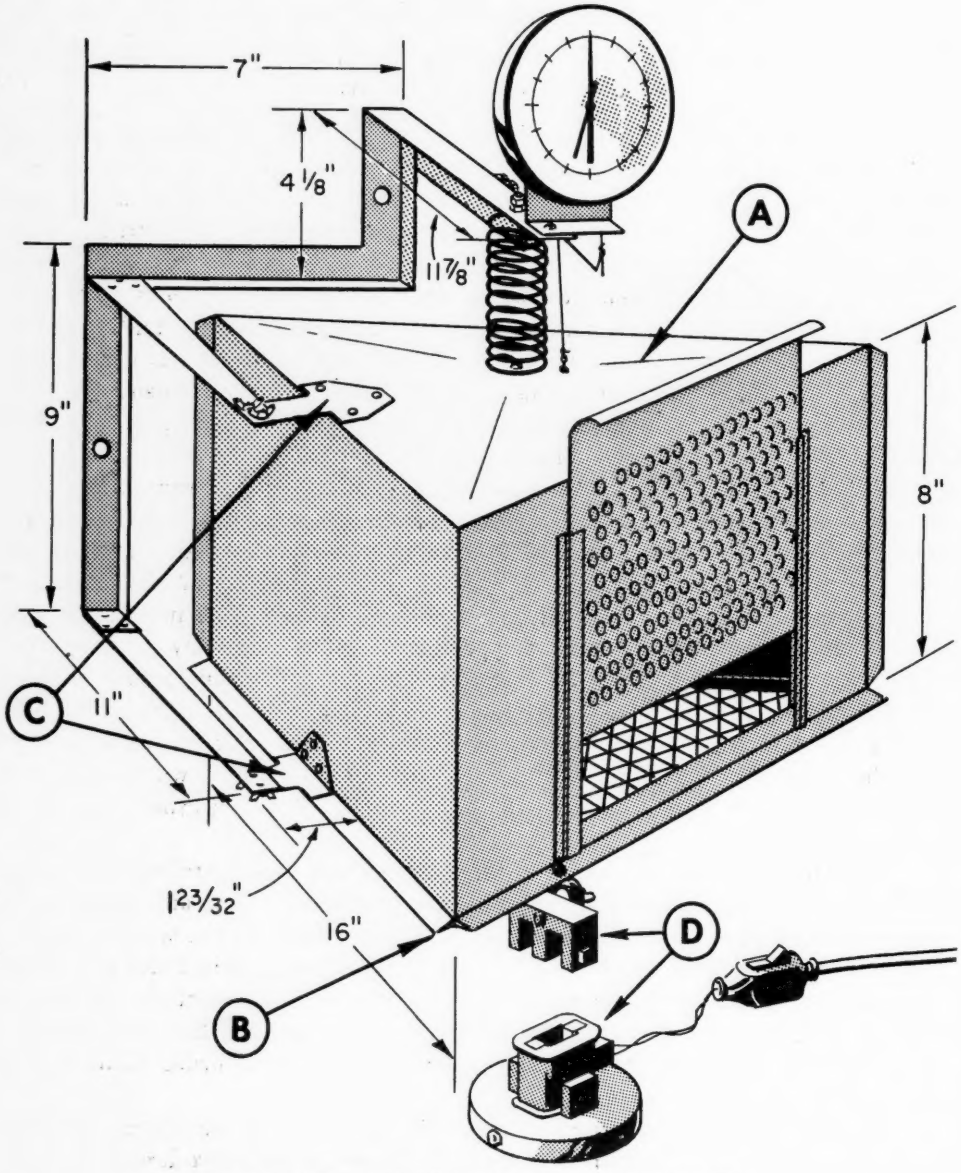

Fig. 1.—Diffuse activity apparatus and calibrator unit.

electrical input. Consequently, a unit was designed and built which operates through a purely mechanical principle. Forty-five of these units have been in constant use for approximately one year and have been found to satisfy the previously described instrument requirements.

APPARATUS

The apparatus is composed of four primary components: (1) the cage, (2) the suspension assembly, (3) the watering system, and (4) the integrator assembly.

Cage.—The cage is triangular in shape (Fig. 1) and weighs approximately 1,100 grams. The top and sides are made of a single piece of 0.04-inch sheet aluminum which, incidentally, provides excellent visual isolation of the animals. In order to impart rigidity, the center of the top is made approximately 5/16 inch higher than the sides and the aluminum is bent so that the angles formed by the three corners are bisected (Fig. 1, *A*). The bottom is made of galvanized steel 14 gauge, ½-inch wire mesh and is held in place by crimping the aluminum siding (Fig. 1, *B*). The 6- by 8-inch access opening is covered by a sliding aluminum door which is perforated to decrease air resistance to the unit. Two aluminum stabilizer attachments are riveted to one side of the cage (Fig. 1, *C*).

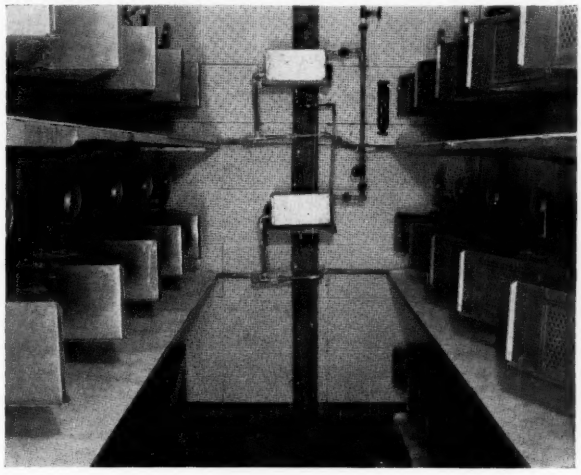

Fig. 2.—Diffuse activity room.

Suspension Assembly.—The suspension assembly is composed of three parts: (1) the bracket, (2) the stabilizers, and (3) the coil spring. The bracket is made of ⅛-inch iron. Two identical stabilizers, made of 0.03-inch phosphor bronze which taper in width from 1¼ to ⅝-inch, are riveted to the bracket. These stabilizers prevent both lateral and rotational displacements of the cage and, in addition, act as dampers to prevent excessive oscillation of the cage. The coil spring by which the cage is suspended from the bracket is made of 0.08-inch-diameter tightly coiled phosphor bronze wire. There are 10 coils measuring 1⅜ inches outside diameter.

Watering System.—An automatic watering device is utilized since frequent replenishment of small quantities of water would unduly disturb the animals and the use of containers of large capacity would change the suspended mass appreciably over long periods of time. Reservoir tanks are connected to the building water supply by intake float valves, and the water is gravity-fed through pipes and plastic tubing to the cages (Fig. 2). The length of tubing between the wall and the cage is kept constant since the tubing moves with the cage. The watering system in the cage consists of ⅛-inch pipe fittings terminating in a fount valve. It is extended sufficiently into the cage to provide a water supply that is easily accessible even to animals made asthenic through experimental procedures.

Integrator.—The integrator (Figs. 3 and 4) is made of modified standard clock parts and summates the amount of the downward displacements of the cage. The clock parts consist of six spur gears and four spacers. The gear arrangement is such that, when Gear A is

revolved 10 times by the pulley, Gear B revolves once causing the large dial hand to revolve by direct drive. When Gear B is revolved 10 times, Gear C revolves once and when Gear C is revolved 10 times, Gear D revolves once causing the smaller dial hand to make one revolution. As a consequence, for every 10 revolutions of the pulley (E), the larger dial hand revolves once and the smaller dial hand makes 1/100 revolution. The integrator is activated by a mercerized cotton string (Fig. 3, *C*) which is tied to the top of the cage, passed over the pulley, and attached to a straight wire spring (Fig. 3, *A*) 5 inches in length and made of 0.025-inch phosphor bronze wire. To prevent reverse movements of the dial hand on the upward displacement of the cage, a second string, which acts as a brake, is tied to the dial stand, passed over the pulley, and attached to a second straight wire spring (Fig. 3, *B*). The pulley over which both strings are placed is made of hollowed aluminum and has an effective diameter of 0.70 inch. The ends of the straight wire springs are looped around the bolt furthest from the integrator. The springs pass beneath the bolt closest to the integrator at an angle, prior to string attachment, of approximately 45 degrees from the horizontal and are held in place by both bolts.

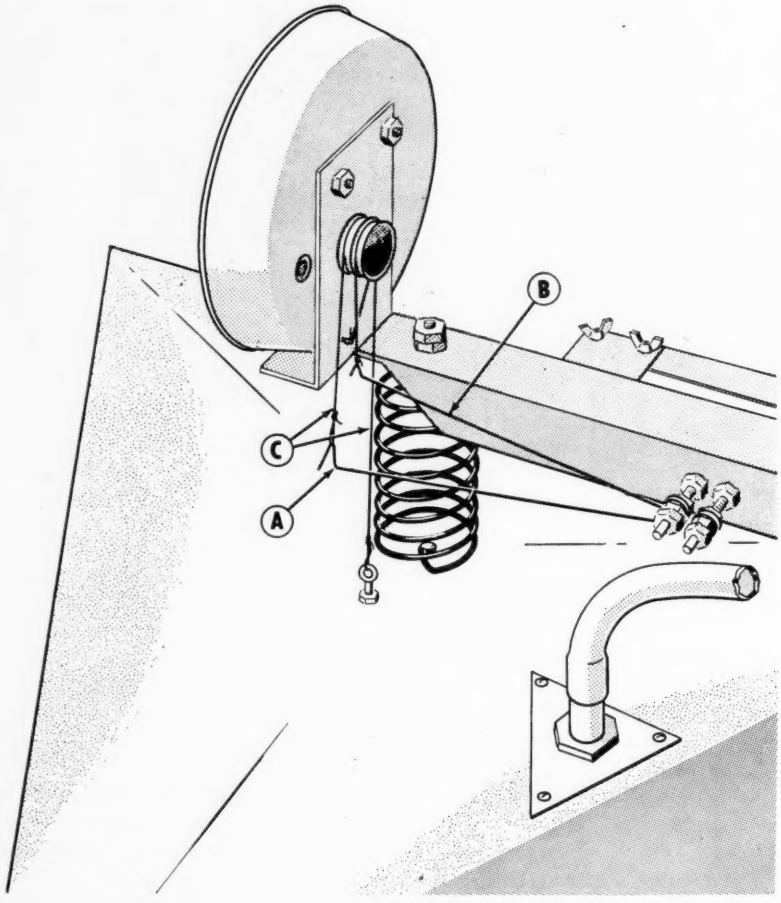

Fig. 3.—Integrator assembly showing string arrangement.

Adjustment of Unit.—A mass corresponding to the maximum load anticipated during the period of observation is added to the cage and the string lengths are adjusted so that

the springs are essentially horizontal. Minor adjustments of the string lengths and/or spring angles are made to ensure satisfactory responses of the integrator to both slight and vigorous movements of the empty and of the loaded cage.

Calibration of Unit.—A calibrator (Fig. 1, *D*) provides a standard reproducible procedure for imparting motion to the cage. The calibrator is used for evaluating the comparative sensitivity of response among units as well as for checking the reliability of response with prolonged use of a unit. The calibrator is a shaded pole electromagnet weighted with a lead plate. The over-all weight of the calibrator is 1,158 grams.

During calibration, the unweighted portion of the magnet is bolted to the cage, and the weighted part is fitted to it and held in place when the circuit is completed. The circuit is then broken, allowing the weighted portion to fall free. The cage is permitted to oscillate to the rest position and the dial is read.

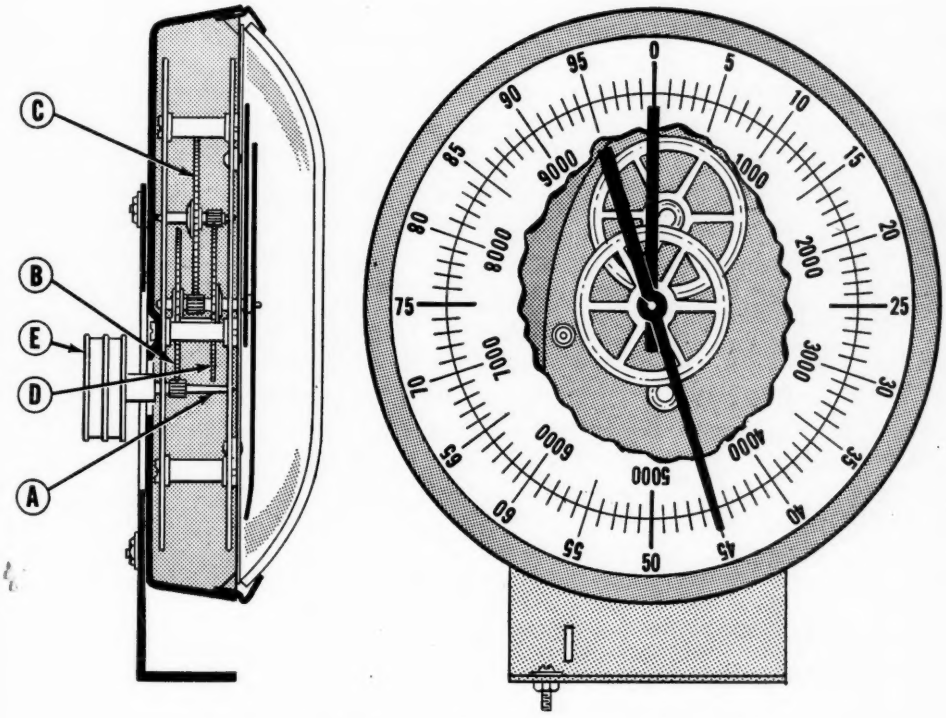

Fig. 4.—Integrator assembly showing gear relationships.

TEST RESULTS

Only one unit has needed major readjustment since the establishment of calibration values for forty-five units approximately one year ago. Infrequently, during the course of an experiment the tension of the straight wire spring (Fig. 3, *A*) of a unit may need to be increased slightly. The values in Table I indicate the degree of variation in the responses of the cages to the calibrator over a period of nine months.

The response of the cages to the calibrator appears to be stable over a relatively wide range of mass changes; e.g., differences in calibration values were

less than 6 per cent upon the addition of 200 Gm. and less than 10 per cent upon the addition of 460 Gm. of mass to the cage. In terms of variability resulting from the position of the mass in the cage, differences of less than 3 per cent were found when a mass (460 Gm.) was placed at various positions on the cage floor.

TABLE I. CALIBRATION VALUES, IN DIAL UNITS, OF 45 CAGES OVER NINE MONTHS

DATE	RANGE OF DIAL READINGS	MEAN DIAL READING	STANDARD DEVIATION
2/ 9/53	80-125	105	9.3
4/22/53	78-129	103	14.6
5/ 1/53	72-121	101	5.1
7/31/53	78-130	105	13.3
11/ 3/53	69-114	95	9.1
11/ 6/53	73-116	96	11.8

Adult male rats were placed in the units in order to determine the reliability of the cage response to the calibrator during eight weeks of usage. The cages were located in a sound-damped, air-conditioned room. Conditions of constant light and relatively constant temperature were maintained throughout the experiment. It was observed that the range of activity of normal rats was 1,000 to 5,000 dial units per 24 hours. Correlation coefficients for an estimation of the reliability of weekly calibrations were found to be 0.89, 0.83, 0.98, 0.91, 0.97, 0.92, and 0.91 among eleven inhabited cages at eight successive weekly intervals. Upon the basis of these figures the cages were deemed reliable for our purposes.

To determine the duration of time which constitutes a reliable estimation of activity, readings were taken for seven-, seventeen-, and twenty-four-hour periods over the course of ten weeks. Rank order correlations (Table II) indicate that a twenty-four-hour period provides the most reliable index of normal activity for the rat.

TABLE II. RANK ORDER CORRELATION COEFFICIENTS OF THE ACTIVITY OF NORMAL ADULT MALE RATS FOR CONSECUTIVE WEDNESDAYS

PERIOD OF MEASUREMENT (HR.)	CORRELATION COEFFICIENTS (WK.)								
	1-2	2-3	3-4	4-5	5-6	6-7	7-8	8-9	9-10
7 9 A.M. to 4 P.M.	−0.10	−0.61	−0.33	−0.02	−0.14	0.18	0.05	0.60	0.63
17 4 P.M. to 9 A.M.	0.89	0.60	0.43	0.32	0.60	0.75	0.73	0.32	0.53
24	0.79	0.91	0.94	0.65	0.85	0.90	0.95	0.98	0.89

SUMMARY

A mechanical apparatus for the measurement of diffuse activity of small animals has been developed. It consists of a spring-suspended, stabilized cage and an integrator, made of modified clock parts, which yields a cumulative, numerical index of the animal's activity. An automatic watering system for use with the unit is described and an instrument for calibrating the unit has been devised.

In a study of its properties the apparatus has been found to be stable in its response despite marked changes in load mass and location of mass within the cage. Rank order correlations of the twenty-four-hour activity values of rats have shown the units to be reliable for the test over a ten-week period of continuous use.

Forty-five of the units have been in almost continuous use for a period of approximately one year and have proved to be satisfactory with respect to the measurement of activity over long periods of time and, in addition, have needed essentially no mechanical adjustments. Although the apparatus has been used with rats and guinea pigs, slight adjustments of the existing parts should make the unit adaptable for use with other small animals.

We wish to express our appreciation to E. Merle Rader, Instruments Branch, U. S. Naval Radiological Defense Laboratory, for his contributions in the design of the original unit.

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Erratum

In the article by A. G. Bearn, M.D., and H. G. Kunkel, M.D., entitled "Metabolic Studies in Wilson's Disease Using Cu^{64}," which appears on pages 623-631 of the April issue of the JOURNAL, on page 630 the second sentence of the final paragraph of text should read as follows: "Prompt fall in urinary radioactivity was noted in normal subjects and was not observed in patients with Wilson's disease."